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**The Study of Acidophilic, Moderately Thermophilic Iron-Oxidizing
Bacteria.**

Darren Alan Clark

Thesis submitted for the Degree of Doctor of Philosophy.

University of Warwick.

Department of Biological Sciences.

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Table of Contents.

Contents	i
List of figures.	vi
List of tables.	xii
Acknowledgements.	xiii
Declaration.	xiv
Abbreviations.	xv
Summary.	xvii

Chapter 1: Introduction.

<i>1.1 Organisms involved in mineral oxidation.</i>	1
1.1.1 The mesophiles.	1
1.1.2 The moderate thermophiles.	2
1.1.3 The extreme thermophiles.	6
<i>1.2 Analysis of mixed microbial populations from natural and commercial bioleaching environments.</i>	7
<i>1.3 Carbon dioxide utilisation.</i>	9
1.3.1 CO ₂ fixation.	9
1.3.2 CO ₂ uptake.	13
1.3.3 Carboxysomes.	15
<i>1.4 Metabolism of Chemolithotrophic Bacteria.</i>	17
1.4.1 Iron oxidation.	17
1.4.2 Sulphur oxidation.	18
1.4.3 Oxidation of sulphide minerals.	19
<i>1.5 Commercial application of bacterial oxidation.</i>	21
1.5.1 Methods employed for bacterial oxidation.	22

	Page
1.5.2 Potentially extractable metals.	24
1.5.3 Thermophiles in bio-oxidation.	29
1.6 <i>Project aims.</i>	33
 Chapter 2: Materials and Methods	
2.1 <i>Bacterial strains.</i>	34
2.2 <i>Chemicals and growth media.</i>	34
2.2.1 Chemicals.	34
2.2.2 Media.	34
2.2.3 Growth substrates and media supplements.	36
2.2.4 Minerals used during leaching studies.	36
2.3 <i>Growth conditions.</i>	37
2.4 <i>Growth assays.</i>	38
2.5 <i>Cell harvesting and lysis.</i>	39
2.6 <i>Polyacrylamide gel electrophoresis (PAGE).</i>	39
2.7 <i>Western blotting.</i>	40
2.8 <i>Densitometer analysis and photography.</i>	41
2.9 <i>Microscopy.</i>	42
2.9.1 Phase contrast microscopy.	42
2.9.2 Transmission electron microscopy.	42
2.10 <i>Percoll gradient centrifugation.</i>	43
2.11 <i>Measurement of iron affinities.</i>	43
2.12 <i>Measurement of CO₂ uptake by whole cells.</i>	44
2.13 <i>Permeabilised whole cell assay for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO).</i>	47
2.14 <i>DNA manipulation techniques.</i>	49
2.14.1 Preparation of chromosomal DNA.	49
2.14.2 Analysis of DNA base composition.	49
2.14.3 DNA hybridisation studies.	50

	Page
2.14.4 PCR amplification of RuBisCO LSU gene fragments.	51
2.14.5 Estimation of DNA concentration.	52
2.15 <i>Whole cell hybridisation with fluorescent-labelled</i>	52
<i>16S rRNA-targeted oligonucleotide probes.</i>	
2.15.1 Probe design.	52
2.15.2 Cell fixation.	55
2.15.3 Slide preparation.	55
2.15.4 Whole cell hybridisation.	55

Chapter 3: Characterisation and Classification of Moderately Thermophilic Iron-oxidizing Bacteria.

3.1 <i>Introduction.</i>	57
3.2 <i>Analysis of DNA:DNA hybridisation values.</i>	59
3.3 <i>Analysis of total cellular protein electrophoretic patterns.</i>	65
3.4 <i>Analysis of DNA base composition.</i>	67
3.5 <i>Morphology of moderately thermophilic iron-oxidizing bacteria.</i>	69
3.5.1 Phase contrast microscopy of vegetative cells.	70
3.5.2 Transmission electron microscopy.	72
3.6 <i>Physiology of moderately thermophilic iron-oxidizing bacteria.</i>	80
3.6.1 Autotrophic growth on ferrous iron.	80
3.6.2 Heterotrophic growth on yeast extract.	82
3.6.3 Growth of moderately thermophilic iron-oxidizing bacteria in the presence of antibiotics.	82
3.6.4 Growth of moderately thermophilic iron- oxidizing bacteria in the presence of metals.	94
3.7 <i>Use of fluorescently labelled oligonucleotides probes complimentary to 16S rRNA.</i>	98
3.8 <i>Discussion.</i>	99

Chapter 4: Characterisation of an Icelandic Mixed Culture.

4.1	<i>Introduction.</i>	103
4.2	<i>Separation of isolates from the mixed culture.</i>	103
4.2.1	Isolation of ICC culture.	104
4.2.2	ICC enrichment cultures.	109
4.2.3	Percoll buoyant density gradients.	113
4.2.4	Isolation of novel strain ICP.	122
4.3	<i>Characterisation of novel isolates ICP and ICH.</i>	124
4.3.1	Analysis of DNA base composition.	124
4.3.2	Analysis of total cellular protein profiles.	125
4.3.3	Morphology of novel isolates.	126
4.3.4	Physiology of novel isolates.	130
4.4	<i>Study of the interactions within the ICC mixed culture.</i>	135
4.4.1	Percoll gradient centrifugation with novel isolates.	135
4.4.2	Autotrophic growth of novel isolates.	138
4.4.3	Affinity of isolate ICP for iron.	141
4.5	<i>Discussion.</i>	151

Chapter 5: CO₂ Utilisation Studies.

5.1	<i>Introduction.</i>	154
5.2	<i>CO₂ uptake.</i>	155
5.2.1	CO ₂ uptake by culture ICC.	155
5.2.2	Requirement of re-suspended cells of culture ICC for a regeneration period.	159
5.2.3	CO ₂ uptake by pure isolates from the ICC culture.	162
5.3	<i>CO₂ fixation.</i>	167
5.3.1	Permeabilised whole cell assay for RuBisCO.	167
5.3.2	Analysis of cell protein profiles of strain ICP under different CO ₂ concentrations.	169

	Page
5.3.3 PCR amplification of RuBisCO LSU gene fragment from strains NAL and ICP.	172
5.4 <i>Discussion.</i>	180
 Chapter 6: Mineral Leaching Studies.	
6.1 <i>Introduction.</i>	184
6.2 <i>Leaching of the Mexican pyrite concentrate.</i>	185
6.3 <i>Leaching of the Bogosu arsenopyrite concentrate.</i>	188
6.4 <i>Leaching of the Olympia arsenopyrite concentrate.</i>	195
6.5 <i>Mineral leaching by an Icelandic moderately thermophilic mixed culture.</i>	201
6.6 <i>Discussion.</i>	206
 Chapter 7: Conclusions.	209
 Bibliography.	213

List of figures

Figure 2.1	Target sequences for strain TH3 16S rRNA specific oligonucleotide probes.	54
Figure 3.1	The effect of filter bound DNA concentration on hybridisation.	60
Figure 3.2	Dendrogram of moderately thermophilic iron-oxidizing bacteria based on DNA:DNA hybridisation values.	62
Figure 3.3	Relationship of organisms on the basis of DNA:DNA hybridisation values.	64
Figure 3.4	Dendrogram of moderately thermophilic iron-oxidizing bacteria based on total cellular protein electrophoretic patterns.	66
Figure 3.5	Phase contrast photomicrographs of three moderately thermophilic iron-oxidizing bacteria.	71
Figure 3.6	Thin section electron micrograph of strain BC1.	73
Figure 3.7	Thin section electron micrograph of strain NAL.	73
Figure 3.8	Thin section electron micrograph of strain ALV.	75
Figure 3.9	Thin section electron micrograph of strain TH3.	75
Figure 3.10	Thin section electron micrograph of strain BC1 showing slight distension of the sporangia during spore formation.	76
Figure 3.11	Thin section electron micrograph of strain NAL showing large distension of the sporangia during spore formation.	76
Figure 3.12	Thin section electron micrograph of strain NAL in early stages of endospore formation.	78
Figure 3.13	Thin section electron micrograph of strain THWX in late stages of endospore formation.	78
Figure 3.14	Thin section electron micrograph showing mature endospore.	79

	Page
Figure 3.15 Iron oxidation during the autotrophic growth of five moderately thermophilic iron-oxidizing bacteria.	81
Figure 3.16 Heterotrophic growth of three moderately thermophilic iron-oxidizing bacteria.	83
Figure 3.17 Zones of growth and iron oxidation inhibition for moderately thermophilic iron-oxidizing isolates in antibiotic disc diffusion assays.	87
Figure 3.18 Zones of growth and iron oxidation inhibition for moderately thermophilic iron-oxidizing isolates in metal diffusion assays.	96
Figure 4.1 Iron oxidation during the autotrophic growth of three moderately thermophilic cultures.	105
Figure 4.2 Iron oxidation during the autotrophic growth of moderately thermophilic strain BC1 and culture ICC.	106
Figure 4.3 Thin section electron micrograph of culture ICC.	108
Figure 4.4 SDS-PAGE of BC1 type isolates from the ICC mixed culture.	110
Figure 4.5 SDS-PAGE of ICC enrichment cultures.	112
Figure 4.6 Position of culture ICC cell bands on four Percoll gradients.	114
Figure 4.7 Location of bands of cells from culture ICC after Percoll gradient centrifugation of a cell suspension.	116
Figure 4.8 Position of cell bands from culture ICC on a 71% Percoll gradient.	117
Figure 4.9 Thin section electron micrograph of cells from the upper and lower bands of a 71% Percoll gradient of culture ICC.	118
Figure 4.10 SDS-PAGE of samples extracted from a 71% Percoll gradient of culture ICC.	120
Figure 4.11 SDS-PAGE of isolates cultured from bands extracted from a 71% Percoll gradient of culture ICC.	121

	Page
Figure 4.12 SDS-PAGE of novel isolates able to grow well autotrophically under air.	123
Figure 4.13 Phase contrast photomicrographs of novel moderately thermophilic strains ICH and ICP.	127
Figure 4.14a Thin section electron micrograph of strain ICH.	129
Figure 4.14b Thin section electron micrograph of strain ICP.	129
Figure 4.15 Heterotrophic growth of novel moderately thermophilic isolates.	131
Figure 4.16 Zones of growth inhibition for novel moderately thermophilic iron-oxidizing isolates ICH and ICP.	132
Figure 4.17 Effect of temperature on the growth of novel isolate ICP during heterotrophic and autotrophic growth.	134
Figure 4.18 Comparison of banding patterns of strains ICH, ICP and the mixed culture ICC on Percoll gradients.	136
Figure 4.19 SDS-PAGE of novel isolates ICH and ICP compared to cell bands extracted from a 71 % Percoll gradient of culture ICC.	137
Figure 4.20 Iron oxidation during the autotrophic growth of four moderately thermophilic iron-oxidizing cultures.	139
Figure 4.21 Iron oxidation during the autotrophic growth of three moderately thermophilic iron-oxidizing bacteria in pure and mixed cultures.	140
Figure 4.22 Iron oxidation during the autotrophic growth of two moderately thermophilic iron-oxidizing bacteria in the presence of ferric iron.	143
Figure 4.23 Lineweaver-Burk double reciprocal plot for oxygen uptake rates of autotrophically grown strain ICP.	144
Figure 4.24 Iron oxidation during autotrophic and chemolithoheterotrophic growth of two moderately thermophilic iron-oxidizing bacteria.	146
Figure 4.25 Lineweaver-Burk double reciprocal plot for oxygen uptake rates of chemolithoheterotrophically grown strain ICP.	147

	Page
Figure 4.26 Lineweaver-Burk double reciprocal plot for oxygen uptake rates of chemolithoheterotrophically grown <i>S. thermosulfidooxidans</i> .	148
Figure 4.27 Lineweaver-Burk double reciprocal plot for oxygen uptake rates of autotrophically grown <i>S. thermosulfidooxidans</i> .	149
Figure 5.1 CO ₂ uptake at various assay CO ₂ concentrations by culture ICC grown autotrophically under 1 % (v/v) CO ₂ in air.	156
Figure 5.2 CO ₂ uptake at various assay CO ₂ concentrations by culture ICC grown autotrophically under air.	157
Figure 5.3 Maximal CO ₂ uptake rates by culture ICC at a range of assay CO ₂ concentrations.	158
Figure 5.4 CO ₂ uptake by re-suspended cells of culture ICC and strain BC1.	160
Figure 5.5 CO ₂ uptake by re-suspended cells of culture ICC and strain BC1 after a four hour incubation period.	161
Figure 5.6 CO ₂ uptake at various assay CO ₂ concentrations by strain ICP grown autotrophically under 1 % (v/v) CO ₂ in air.	164
Figure 5.7 CO ₂ uptake at various assay CO ₂ concentrations by strain ICP grown autotrophically under air.	165
Figure 5.8 Maximal CO ₂ uptake rates by strain ICP at a range of assay CO ₂ concentrations.	166
Figure 5.9 SDS-PAGE followed by anti- <i>T. ferrooxidans</i> RuBisCO western blot of strain ICP grown under different conditions.	168
Figure 5.10 CO ₂ fixed via RuBisCO during a permeabilised whole cell assay with moderate thermophiles ICP and BC1.	170
Figure 5.11 1 % (w/v) agarose minigel of PCR mix with RuBisCO LSU primers.	173

	Page
Figure 5.12 The nucleotide and deduced amino acid sequences of a RuBisCO LSU gene fragment from strains NAL and ICP.	175
Figure 5.13 Comparison of the deduced amino acid sequences of RuBisCO LSU gene fragments from strains NAL and ICP with other organisms.	176
Figure 5.14 Relationship between RuBisCO LSU gene fragments from strains ICP and NAL, and the sequences from other organisms.	183
Figure 6.1 The solubilisation of 2% (w/v) Mexican pyrite concentrate during the growth of three cultures of mineral-oxidizing bacteria.	186
Figure 6.2 The solubilisation of 2% (w/v) Bogosu arsenopyrite concentrate during the growth of three cultures of mineral-oxidizing bacteria.	189
Figure 6.3 The solubilisation of 10% (w/v) Bogosu arsenopyrite concentrate during the growth of three cultures of mineral-oxidizing bacteria.	191
Figure 6.4 The solubilisation of 12% (w/v) Bogosu arsenopyrite concentrate during the growth of <i>Sulfolobus</i> strain BC at 68 °C.	194
Figure 6.5 The solubilisation of 2% (w/v) Olympia arsenopyrite concentrate during the growth of three cultures of mineral-oxidizing bacteria.	196
Figure 6.6 The solubilisation of a mixture of 1% (w/v) Olympia arsenopyrite concentrate and 1% (w/v) Mexican pyrite concentrate during the growth of three cultures of mineral-oxidizing bacteria.	198

	Page
Figure 6.7 The solubilisation of 4% (w/v) Olympia arsenopyrite concentrate during the growth of <i>Sulfolobus</i> strain BC at 68 °C.	200
Figure 6.8 The solubilisation of 2% (w/v) Mexican pyrite concentrate during the growth of three moderately thermophilic cultures at 48 °C.	202
Figure 6.9 The solubilisation of 2% (w/v) Bogosu arsenopyrite concentrate during the growth of two moderately thermophilic cultures at 48 °C.	203
Figure 6.10 The solubilisation of 2% (w/v) Olympia arsenopyrite concentrate during the growth of two moderately thermophilic cultures at 48 °C.	205

List of Tables.

Table 1.1	World production, prices and annual values of some metals.	25
Table 1.2	Cost comparison of pre-treatment plants at Sao Bento, Brazil.	28
Table 2.1	Chemical analysis of mineral concentrates.	37
Table 3.1	Moderately thermophilic iron-oxidizing bacteria.	58
Table 3.2	DNA:DNA hybridisation values of moderately thermophilic iron-oxidizing bacteria.	63
Table 3.3	DNA melting temperatures and base compositions of moderately thermophilic iron-oxidizing bacteria.	69
Table 3.4	Characteristics of antibiotics used in disc assays.	86
Table 4.1	DNA melting temperatures and base composition of novel moderately thermophilic iron-oxidizing bacteria.	124
Table 4.2	Homology values for novel moderately thermophilic iron-oxidizers based on SDS-PAGE patterns.	126
Table 4.3	Apparent K_m and K_i values for ferrous and ferric iron.	150
Table 5.1	Densitometry analysis of putative RuBisCO LSU band from strain ICP.	171
Table 5.2	Comparison of nucleotide and deduced amino acid sequence homologies of RuBisCO LSU gene fragments from strains NAL and ICP with other organisms.	177
Table 5.3	Comparison of the codon usage in a gene fragment of the RuBisCO LSU from strains NAL and ICP	179

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Declaration

I declare that this thesis has been composed by myself, and has not been used in any previous application for a degree. All results except where stated have been obtained by me under the supervision of Dr. Paul Norris at Warwick University, and Mr Lienel Barnes at Shell Research Ltd.

Abbreviations

AMP	Adenosine monophosphate.
ATP	Adenosine triphosphate.
bp	base pairs.
CA	Carbonic Anhydrase.
CCM	Carbon Concentrating Mechanism.
C _i	Single carbon compounds.
Ci	Curies.
cpm	counts per minute.
°C	degrees Celsius.
DNA	Deoxyribonucleic acid.
\$	dollars.
EA	Enzyme Activity.
Fig.	Figure.
x g	gravitational force.
G+C	Guanosine + cytidine.
g	grams.
kg	kilograms.
kDa	kilodaltons.
K _m	Michaelis constant.
K _i	Inhibitor constant.
LSU	Large Subunit.
l	litres.
ml	millilitres.
min	minutes.
mol	moles.
M	Molar.
mM	milliMolar.
μM	microMolar.
mg	milligrams.
μg	micrograms.
μm	micrometre.
nm	nanometres.
NADH	Nicotinamide Adenine Dinucleotide (reduced form).
OD	Optical Density.
PRK	Phosphoribulokinase.
rpm	revolutions per minute.

RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase.
RuBP	Ribulose-1,5-bisphosphate.
rRNA	ribosomal Ribonucleic acid.
s.	substrate concentration
SA	Specific Activity.
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.
SSU	Small Subunit.
T _m	Melting temperature.
UPGMA	Unweighted Pair Group Method Using Arithmetic Averages.
n	reaction velocity.
v/v	volume per volume.
w/v	weight per volume.
>	greater than.
<	less than.

Summary

Acidophilic, mineral sulphide-oxidizing bacteria are being used commercially in reactors for processing gold-bearing minerals. Bacterial activity may limit the cost effectiveness of these processes. Use of thermophiles could reduce process times and reactor cooling costs in comparison with current applications of the mesophile *Thiobacillus ferrooxidans*. Among these organisms, the apparently variable efficiency of carbon dioxide utilisation has been studied as an example of a factor in organism selection in relation to process optimisation.

This study has divided the most frequently isolated types of moderate thermophiles into three groups: isolates of *Sulfobacillus thermosulfidooxidans* (mol% G+C 47-50), an isolate referred to as strain NAL and other closely related species (mol% G+C 54-57), and the type previously referred to as strain TH3 (mol% G+C 68). These three groups differ both morphologically and physiologically.

In contrast to *T. ferrooxidans* which grows well in the absence of enhanced carbon dioxide concentrations, all previously studied moderate thermophiles required levels of carbon dioxide above those present in air for autotrophic growth. An enrichment culture was obtained that could efficiently solubilise a range of mineral sulphides at 48 °C under air. Characterisation of this culture indicated the presence of two organisms essential for efficient growth under air: a typical *S. thermosulfidooxidans* group organism (isolate ICH), and a strain TH3 group organism (isolate ICP). Strain ICP appeared to possess an inducible, high affinity transport system for carbon dioxide during growth under air (unlike any previously studied moderate thermophiles), but extensive oxidation of ferrous iron was not achieved even at enhanced carbon dioxide levels. This lack of oxidation appeared to be the result of autotrophically-grown strain ICP having an apparent higher affinity for the end-product of iron oxidation, ferric iron (K_i 0.4 mM), than the substrate, ferrous iron (K_m 0.5 mM). Only when a mixed culture of strain ICP and strain ICH was grown did extensive oxidation occur, indicating a commensal relationship between these two isolates.

A DNA fragment (390 bp) encoding part of the RuBisCO LSU gene (amino acids 70-200) was amplified from the genomic DNA of moderate thermophile strains ICP and NAL using PCR. A comparison of the hexadecameric LSU sequences from a range of organisms indicated that the RuBisCO LSU gene fragment from strain ICP was most closely related to that of the iron-oxidizing autotroph *T. ferrooxidans* (93.8% amino acid identity), whilst that of strain NAL appeared most closely related to the sequence of moderate thermophile NMW-6 (82.2% amino acid identity). These differences in sequence homology suggest that the evolution of the RuBisCO genes from strains NAL and ICP diverged at an early stage.

A comparative mineral leaching study, with a mesophilic, a moderately thermophilic, and an extremely thermophilic culture indicated that the moderately thermophilic culture was the most robust during the dissolution of a range of minerals. This culture gave consistently better mineral dissolution rates than the mesophilic culture, clearly indicating their immediate commercial potential. In comparison the extremely thermophilic culture often produced faster rates of mineral dissolution than the moderately thermophilic culture, but appeared sensitive to agitation at high mineral pulp densities (10% (w/v)), limiting any present commercial applications of these organisms.

Chapter 1:
Introduction.

Bio-oxidation can be used to solubilise mineral sulphide ores, to release their metal values (Barrett *et al.*, 1993). The industrial application of bio-oxidation has now been extended passed the relatively crude heap and dump operations used for copper recovery (Torma, 1993), to the extraction of gold from refractory ores using bioreactors (van Aswegen, 1993). These bioreactors enable greater process control, and hence a more efficient process. The cost effectiveness of these processes may be enhanced further by overcoming limitations to the bacterial activity. However, identifying targets for strain improvement is difficult, because relatively little characterisation of the acidophilic mineral sulphide-oxidizing bacteria involved in bio-leaching has been carried out. Previous studies have indicated some factors that are likely to be of importance in organism selection in relation to process optimisation. One of these factors is the apparently variable efficiency of carbon dioxide utilisation.

1.1: Organisms involved in mineral sulphide oxidation.

Thiobacillus ferrooxidans was the first bacterium shown to be capable of oxidizing sulphide minerals (Colmer *et al.*, 1950). Until 1972 this was considered to be the only organism important in the leaching of minerals. Since then a large number of physiologically diverse bacteria have been isolated that are capable of mineral sulphide oxidation. A convenient way of describing these bacteria is by reference to their optimal temperatures for growth. Three groups of bacteria can be considered; the mesophiles (optimum temperatures about 30 °C), the moderate thermophiles or thermotolerant bacteria (optimum temperatures about 50 °C), and the extreme thermophiles (optimum temperatures about 70 °C).

1.1.1: The mesophiles.

This group contains the most studied mineral sulphide-oxidizing bacterium, *Thiobacillus ferrooxidans*. *T. ferrooxidans* appears to be the most dominant organism in mineral sulphide oxidation below 40 °C. It is a Gram-negative, rod-shaped, acidophilic,

obligate chemolithoautotroph, with dimensions of 0.3-0.5 μm x 1-1.7 μm that is capable of both iron and sulphur oxidation (Barrett *et al.*, 1993). Cells usually appear singly or in pairs and sometimes possess a polar flagellum. The growth temperature range for most strains is between 15-37 °C, with an optimum around 30 °C. Exceptions to this temperature range do exist (Ferroni *et al.*, 1986). Optimal growth and iron oxidation occur around pH 2.0 (Leduc and Ferroni, 1994). There are a large number of strains of *T. ferrooxidans*, which are genomically more diverse than is customary for a single species. At least five DNA:DNA hybridisation groups have been identified (Harrison, 1982). DNA base composition is normally about 57-59 mol% G+C. Grouping on the basis of partial 16S rRNA sequences placed most *T. ferrooxidans* strains in the beta sub-division of the Proteobacteria (Lane *et al.*, 1992).

Thiobacillus thiooxidans resembles *T. ferrooxidans* in many ways, but differs in its inability to oxidize iron, and it has a lower G+C mol% base composition (Harrison, 1982). In pure culture *T. thiooxidans* does not effectively degrade pyrite, but can contribute to dissolution of some minerals when in mixed cultures.

Leptospirillum ferrooxidans is an obligate chemolithoautotroph that can only oxidize iron. *L. ferrooxidans* is readily distinguishable from *T. ferrooxidans*, as cells are slightly thinner, vibrioid and often develop into spiral forms (Norris, 1989a).

Other bacteria that are often found associated with *T. ferrooxidans* include acidophilic heterotrophs such as *Acidiphilium cryptum*, and mixotrophs such as *Thiobacillus acidophilus* (Harrison, 1984). These heterotrophs and mixotrophs are often closely associated with the autotrophs in a commensal relationship, resulting in some misunderstandings when mixed cultures have been incorrectly assumed pure (Harrison, 1984).

1.1.2: The moderate thermophiles.

The presence of moderately thermophilic, acidophilic bacteria in thermal springs and ore deposits was first demonstrated in 1977 (Brierley and Lockwood, 1977; Le Roux *et al.*, 1977; Golovacheva and Karavaiko, 1978). Several moderately

thermophilic bacteria have been isolated, but identification and classification is not well advanced. The moderate thermophiles have a wider nutritional range than the obligate chemolithoautotrophic *T. ferrooxidans*; being capable of autotrophic, chemolithoheterotrophic, and mixotrophic growth. They have been described as preferentially mixotrophic, with the best growth rates occurring on iron in the presence of organic substrates (Wood and Kelly, 1984). They can oxidize iron and sulphur, but some appear to grow less readily on sulphur and tolerate less acid than the acidophilic thiobacilli (Norris *et al.*, 1986). The moderate thermophiles also differ from the mesophiles in being Gram-positive. Analysis of the DNA base composition of five moderate thermophiles indicated that they represented more than a single genus with G+C values ranging from 50-68.5 mol% (Harrison, 1986).

To date only a single genus, *Sulfobacillus*, has been characterised. The genus *Sulfobacillus* consists of aerobic, facultatively autotrophic, acidophilic, spore-forming bacteria. Three isolates have so far been placed into this genus. All three of these isolates originated from the Soviet Union. The first isolate to be named was *Sulfobacillus thermosulfidooxidans* (type strain VKM B-1269). This bacterium was isolated in 1976 from the Nikolaev sulphidic ore deposit (Golovacheva and Karavaiko, 1978). It shows polymorphism, but the typical cell type is a rod of dimensions 0.6-0.8 x 1-3 μm (Golovacheva, 1979). Spherical spores with dimensions 0.7 x 0.7-0.8 μm are formed terminally or sub-terminally. Its optimal growth temperature is about 50 °C, and its DNA base composition is 47.2 mol% G+C.

A further two sub-species of *S. thermosulfidooxidans* have been named, *S. thermosulfidooxidans* sub-species *thermotolerans* (Kovalenko and Malakhova, 1983), and *S. thermosulfidooxidans* sub-species *asporogenes* (Vartanyan *et al.*, 1989). These sub-species show differences in their morphology and physiology (Karavaiko *et al.*, 1988). The main differences being *S. thermosulfidooxidans* sub-species *thermotolerans* has a lower optimum temperature than the other two strains, (37-42 °C), whilst *S. thermosulfidooxidans* sub-species *asporogenes* does not produce spores.

The morphology of all three strains resembles *Bacillus*, but cell branching and the formation of complex aggregations are similar to those seen with coryneform bacteria (Karavajko *et al.*, 1990). G+C content of the three strains ranges from 45.5-49.3 mol%.

A number of isolates, including strains TH1 and BC1, from various sites around the world resemble *S. thermosulfidooxidans*, although direct comparison has not been carried out. Protein banding patterns obtained by whole cell electrophoresis of some of these isolates show only minor differences (Marsh and Norris, 1983), suggesting that this bacterial type is widespread. Morphology varies depending on the growth conditions. Typical dimensions when grown on ferrous iron and yeast extract are 0.8 x 1.6-6.0 μm . The bacteria are usually smaller when grown autotrophically on iron (Norris *et al.*, 1986). Elongated forms can occur under stress conditions such as high temperatures. Such elongated forms have also been observed during growth on minerals. In common with *S. thermosulfidooxidans* they appear unable to utilise sulphate, and so must be supplied with a reduced sulphur source (Norris and Barr, 1985). Mineral sulphides are adequate for this requirement, as are sulphur containing amino acids such as cysteine. Strains TH1 and BC1 have very similar DNA base compositions, 50 and 49.5 mol% G+C respectively (Harrison, 1986).

Other iron-oxidizing moderate thermophiles have been isolated from a number of sites around the world. The distribution of particular types remains to be examined. Strain ALV, was isolated from a coal spoil tip in Warwickshire, and does not require a reduced sulphur source during autotrophic growth on ferrous iron (Norris and Barr, 1985). It has a higher G+C content than strains BC1 and TH1 at 56-57 mol%. Analysis of partial 16S rRNA sequences indicated close grouping of strain ALV to strain BC1 near the base of the low G+C sub-division of the Gram-positive bacteria, but affiliation to a specific group could not be made (Lane *et al.*, 1992). When grown autotrophically on ferrous iron strain ALV appears almost filamentous (Harrison, 1986), but has a more typical rod shape during chemolithoheterotrophic growth. During mixotrophic

growth glucose is oxidized primarily by the oxidative pentose-phosphate pathway (Wood and Kelly, 1984).

Strain TH3 was originally isolated from a copper leach dump at the Chino mine, Hurley, New Mexico (Brierley, 1978), but was not maintained in a culture collection. An apparently identical strain was isolated from the same site several years later (Norris and Barr, 1985). Cells are smaller ($0.5\ \mu\text{m} \times 1\text{--}1.6\ \mu\text{m}$) than those of strain TH1, and usually grow in a filamentous manner. Motility has been observed, especially during heterotrophic growth. Endospores are never seen. Strain TH3 differs from both strains BC1 and ALV on the basis of 16S rRNA sequences and DNA base composition. Strain TH3 was firmly affiliated with the high G+C sub-division of Gram-positive bacteria on the basis of 16S rRNA (Lane *et al.*, 1992). This affiliation was reflected in the high G+C content of 68.5 mol% (Harrison, 1986). Autotrophic growth on ferrous iron was reported for the original TH3 isolate (Brierley and Brierley, 1986), but the newer isolate appeared unable to grow well without the addition of yeast extract (Norris and Barr, 1985).

Isolate HPTH, originating from an Australian coal mine (Ghauri and Johnson, 1991), had several similarities to strain TH3, but the organisms were not directly compared, and HPTH was not maintained in culture collections. HPTH and strain TH3 showed similar morphology; HPTH had dimensions $0.4\ \mu\text{m} \times 2.4\ \mu\text{m}$, and grew filamentously. The DNA base composition of 68 mol% G+C was also similar to strain TH3. Motility was not reported for HPTH, however, endospore formation was.

Two other moderately thermophilic isolates were described by Ghauri and Johnson (1991), THWX and YTF1. THWX was isolated from a coal slag pile in Wrexham, North Wales, and YTF1 from an acidic thermal spring, Yellowstone National Park, Wyoming, USA. These two isolates were morphologically and physiologically similar to strain TH1, but their reported DNA base compositions were 43 mol% G+C for THWX, and 63 mol% G+C for YTF1.

A mixed thermophilic culture known by the code name M4 has been described (Nobar *et al.*, 1988). This culture consisted of two differently sized rods and a coccus.

The culture had an optimum temperature of 40-45 °C, and was greatly inhibited at 50 °C. The two rods were both Gram-negative, one was an iron-oxidizer and the other a sulphur-oxidizer. The coccus was a Gram-positive iron-oxidizer. These three organisms appeared to remain associated during growth on many mineral sulphides.

A moderately thermophilic strain of *Leptospirillum* has been reported (Golovacheva *et al.*, 1992). It has an optimum temperature for growth of 45-50 °C, and has been named *L. thermoferrooxidans*.

There are a number of thermotolerant sulphur oxidizers. Strain BC13 is a Gram-negative rod. It oxidizes sulphur more rapidly at 45-50 °C, than the Gram-positive iron- and sulphur-oxidizers, such as strain ALV (Norris *et al.*, 1986). The thermotolerance and higher G+C content (50 mol%) suggest that it is a separate species to *T. thiooxidans*. Strain BC13 has recently been characterised and named as the novel species *Thiobacillus caldus* (Hallberg and Lindström, 1994).

All the moderate thermophiles known at present have a maximum growth temperature of 60 °C or below. They also share a requirement for CO₂ levels above those found in air for optimal autotrophic growth (Marsh and Norris, 1983).

1.1.3: The extreme thermophiles.

This group consists of members of the Archaea. Species of *Sulfolobus*, *Metallosphaera*, and *Acidianus* are all capable of mineral sulphide oxidation. They are aerobic, extremely thermophilic and acidophilic cocci (Barrett *et al.*, 1993). There is believed to be a wide range of mineral oxidation activity and considerable phylogenetic diversity amongst these morphologically similar bacteria (Norris, 1989a). Some are facultative chemolithoautotrophs, capable of mixotrophic and heterotrophic growth. *Sulfolobus acidocaldarius* and *Acidianus brierleyi* have pH optima between 1.5-3.0, and grow in the range 55-85 °C and 45-75 °C respectively. DNA base composition amongst species of *Sulfolobus* and *Acidianus* ranges from 31-39 mol% G+C, whilst *Metallosphaera sedula* has a higher mol% G+C content of about 45. CO₂ fixation is believed to be via a reductive carboxylic acid cycle (Fuchs, 1989) rather than via the

Calvin cycle. More recently cultures have been reported that oxidize mineral sulphides well above 70 °C (Norris and Owen, 1993). The extreme thermophiles are reported to be more sensitive to agitation at high solid concentrations than the eubacterial mineral sulphide oxidizers (Lindström and Gunneriusson, 1990). This may be a limitation to their commercial application. However, there are indications that some strains possess adequate tolerance of agitation and high mineral concentrations (Norris and Owen, 1993).

1.2: Analysis of mixed microbial populations from natural and commercial bioleaching environments.

Having introduced the major groups of organisms likely to be responsible for mineral sulphide dissolution, some reference should be made to the ecology of the leaching environment. Although few ecological studies have been carried out, habitats containing mineral sulphides, such as some coal spoil tips, contain a wide range of organisms including fungi and protozoa, as well as the iron- and sulphur-oxidizing bacteria (Harrison, 1978; White, 1983). However, chemosynthesis by the autotrophic mineral-oxidizers is likely to be one of the key factors in this complex ecological system. In commercial bioleaching operations mixed microbial populations inevitably exist given the nature of the culture conditions (e.g. un-sterilised heterogeneous substrates, the use of drainage water, fluctuating temperatures, pH, and oxygen and nutrient availabilities).

The oxidation reactions that occur during mineral dissolution are exothermic. If cooling is not achieved then temperatures well above ambient can be reached. This may lead to a natural succession from mesophilic organisms to thermophilic organisms. For example, an increase in temperature to 45 °C and above within a copper leaching system resulted in the loss of *T. ferrooxidans*, and the development of moderately thermophilic populations (Brierley and Lockwood, 1977).

The identification of mineral oxidizing bacteria within this environment can be difficult, with the poor growth of many strains on solid media making isolation difficult (Johnson and McGinness, 1991). Genetic probes have been suggested as a method of identifying different species within bio-mining operations (Yates *et al.*, 1986). More recently the identification of single bacterial cells from a range of natural environments using radio-, enzyme-, or fluorescently-labelled probes has been described (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Zarda *et al.*, 1991). Labelled oligonucleotide probes between 15-25 nucleotides in length targeted to complementary sites on the 16S rRNA molecule can differentiate between single mismatches in target and probe sequences (Amann *et al.*, 1990a).

Over the past decade extensive 16S rRNA sequence databases have been developed (Olsen *et al.*, 1991). These databases have been used to identify specific signature sequences within the molecule that can be used to differentiate between groups of organisms. Signature sequences for kingdoms, down to species and sub-species levels have all been identified and successfully used (DeLong *et al.*, 1989; Amann *et al.*, 1990b; Hahn *et al.*, 1993).

The method can be developed to take advantage of the high number of ribosomes within a cell during exponential growth. Sufficient target sequences are thus available to allow the fluorescence of single cells to be observed microscopically. Oligonucleotide probes are labelled at the 5' end with a single fluorescent dye (or enzyme) molecule. The cells are permeabilised, and stabilised to allow free penetration of the probe whilst maintaining morphological integrity. After hybridisation the target cells can be identified microscopically.

The technique allows rapid identification without the need for cultivation, and so fastidious or difficult to culture organisms can be demonstrated in their natural environments. Ruminal bacteria (McSweeney *et al.*, 1993), methylotrophic bacteria (Tsien *et al.*, 1990), and sulphate reducing bacteria (Kane *et al.*, 1993) have all been identified in this way. Problems can occur with limited cell permeability, low cellular ribosome contents, auto-fluorescence, and background fluorescence (Amann *et al.*,

1992; Hahn *et al.*, 1992). For example, soil bacteria could not be identified without the addition of nutrients to the soil to increase the growth rate (Hahn *et al.*, 1992). However, the soil particles and minerals did not interfere with the fluorescence, suggesting that the process may be of use in bio-oxidation systems in the future.

1.3: Carbon dioxide utilisation.

For an economical process, bio-oxidation must be carried out by organisms growing autotrophically. Studies of gas mass transfer using thermophilic cultures indicate that carbon dioxide limitation is more likely to occur than oxygen limitation (Boogerd *et al.*, 1990). This makes efficient CO₂ utilisation even more important for thermophiles. The importance of CO₂ utilisation is further emphasised by the incentive to operate bio-oxidation processes at higher solid concentrations, where gas mass transfer can become limiting (Bailey and Hansford, 1993).

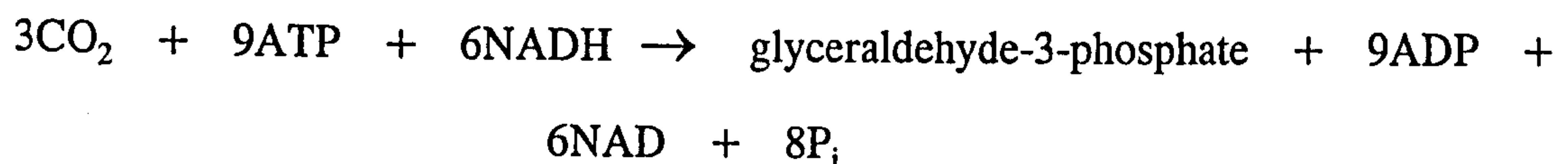
All the moderately thermophilic iron-oxidizers so far studied have required enhanced CO₂ levels for autotrophic growth on minerals, unlike the extreme thermophiles or *T. ferrooxidans* (Norris, 1989b). This requirement for enhanced CO₂ could add costs to commercial application of moderate thermophiles, unless carbonates were used (as they are in gold bio-extraction) to control pH. A moderately thermophilic culture with improved CO₂ utilisation capacities would guarantee a simplified and cheaper process, and therefore be of potential commercial value.

1.3.1: CO₂ fixation.

There are three main pathways of CO₂ fixation in bacteria. The most common pathway is the reductive pentose phosphate cycle, or Calvin cycle (Bowien and Leadbeater, 1984). The two main alternative pathways both utilise acetyl CoA as an intermediate. These alternative pathways are the reductive carboxylic acid cycle, and the reductive acetyl CoA pathway (Fuchs, 1989). The mineral sulphide-oxidizing Archaea probably utilise the reductive carboxylic acid cycle (Norris, 1989b), whilst the

moderately thermophilic mineral sulphide-oxidizers use the Calvin cycle as demonstrated by the activity of some of the key enzymes (Wood and Kelly, 1985). Only the Calvin cycle will be considered further.

The net effect of the Calvin cycle is the production of a C3 compound from CO₂ as follows:



The Calvin cycle consists of 13 reactions, 9 of which are common to both autotrophic and heterotrophic carbon metabolism. The four enzymes unique to the Calvin cycle are; ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), phosphoribulokinase (PRK), fructose-1,6-bisphosphate/sedoheptulose-1,7- bisphosphate aldolase (ALD) and fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase (FBPase/SBPase). PRK and RuBisCO are generally considered the key enzymes whose presence indicates the operation of the Calvin cycle within an organism (Bowien, 1989). Most investigations have focused on these two key enzymes.

PRK.

PRK catalyses the phosphorylation of ribulose 5-phosphate with ATP to yield the CO₂ acceptor, ribulose 1,5-bisphosphate. NADH is a strong activator, and both AMP and phosphoenolpyruvate are inhibitors of the enzyme (Bowien and Leadbeater, 1984). The prokaryotic native enzyme has a molecular weight between 220-256 kDa (Tabita, 1988), and consists of eight identical sub-units of molecular weight 33 kDa (Siebert and Bowien, 1984). The eukaryotic PRK's are dimers of sub-units 41-46 kDa in size (Bowien, 1989). The enzymes from prokaryotes and eukaryotes are fundamentally different in both structure and catalytic properties (Tabita, 1988).

RuBisCO.

More is known about RuBisCO than PRK. RuBisCO has been described as the most abundant protein on earth (Ellis, 1979). The enzyme is in fact a very poor catalyst, with a turnover number of between 1 000 to 2 000 mol CO₂ fixed per mol enzyme per minute (Tabita, 1994). This low value means that a large amount of the enzyme is needed to enable an organism to exist on CO₂ as its sole carbon source.

RuBisCO is a dual function enzyme catalysing both the reduction of CO₂ and the oxidation of ribulose-1,5-bisphosphate (RuBP). RuBisCO from every source including anaerobic bacteria is capable of this dual function (Tabita, 1981). To catalyse either of these reactions the enzyme must first be activated at a site other than the catalytic site. This activation occurs in two steps. An activator CO₂ molecule is carbamylated to the ϵ -amino group of a lysine residue. This is then stabilised by the binding of a divalent cation. The metal specificity for the two functions of the enzymes appears to be different. The carboxylase activity of the enzyme has preference for Mg²⁺, whilst the oxygenase activity is more active with Mn²⁺.

The typical form of RuBisCO is conserved within a variety of diverse species of bacteria, and higher plants. The native enzyme has a molecular weight of 500-600 kDa, and consists of eight large sub-units (55 kDa) and eight small sub-units (15 kDa) (Tabita, 1994). RuBisCO from *Rhodospirillum rubrum* has a unique structure, being a dimer of large sub-units only (Miziorko and Lorimer, 1983). A number of *Rhodobacter* species contain two forms of RuBisCO: form I is the typical L₈S₈ form, whereas form II is smaller, consisting of only large sub-units, and has the structure L_n where n can be anything from 2-8 (Gibson and Tabita, 1985). The two forms of the enzyme in *Rhodobacter sphaeroides* are distinct both immunologically (Gibson and Tabita, 1977), and in primary protein structure (Gibson and Tabita, 1985). Form II RuBisCO has also been reported in other bacteria (Torres-Ruiz and McFadden, 1987; English *et al.*, 1992; Chung *et al.*, 1993).

The large sub-unit (LSU) of RuBisCO contains the active site for both carboxylase and oxygenase activity (Miziorko and Lorimer, 1983). LSUs of form I

RuBisCO from all oxygen evolving photosynthesisers exhibit > 80% homology to one another. Only lys 201 (plant enzyme numbering) has been assigned a function. This is where the activator CO₂ binds. Lys 166 (*R. rubrum* numbering) is also essential for full activity, but its exact function is not known (McFadden, 1989).

The exact function of the small sub-unit (SSU) is not known. Mild dissociation experiments indicate that the SSUs are essential for full enzyme activity (Andrews and Ballment, 1983), but some forms of the enzyme function without small sub-units (English *et al.*, 1992). Three major invariant regions have been identified in SSUs from a diverse range of species, an area near the N-terminus, one in the centre of the primary structure, and a third near the C-terminus (Tabita, 1994). Structural studies (Schneider *et al.*, 1990) indicate that the SSU may modulate substrate binding to the active site on the LSU, by inducing conformational changes. Mutant studies on recombinant cyanobacterial RuBisCO appear to support these structural studies (Tabita, 1994).

The RuBisCO LSU and SSU genes from *T. ferrooxidans* have been cloned and sequenced (Kusano *et al.*, 1991a; Pulgar *et al.*, 1991). Despite two sets of the RuBisCO structural genes being found (Kusano *et al.*, 1991b) both appear to code for the typical form I (L₈S₈) enzyme. The nucleotide and deduced amino acid sequences for this enzyme showed greatest homology to RuBisCO from the photosynthetic bacterium *Chromatium vinosum*. The only other information available about RuBisCO primary structure in iron-oxidizing chemolithoautotrophs is the partial sequence (amino acid residues 73 to 200) for the LSU gene from the moderate thermophile NMW-6 (Holden and Brown, 1993). The partial nucleotide and deduced amino acid sequences showed greatest homology to the chemoautotroph *Alcaligenes eutrophus*. RuBisCO has been reported in the thermophilic hydrogen-oxidizing autotroph *Bacillus schlegelii* (Mikulik *et al.*, 1992), but no sequence data is available.

Regulation of Calvin cycle enzymes.

Facultative autotrophs show a much higher degree of regulation of Calvin cycle enzymes than obligate autotrophs (Smith and Hoare, 1977; Bowien and Schlegel, 1981). In photosynthetic organisms light plays an important role in the regulation of the Calvin cycle, and in chemolithotrophs the enzymes are modulated by metabolic effectors (Crawford *et al.*, 1984).

The main metabolic signals for regulation are the energy state of the cell and the availability of reducing power. This is seen in the inhibition of PRK by AMP and in its activation by NADH. End-product inhibition probably also plays a part, with maximal de-repression when CO₂ is limiting, and maximum repression when organic compounds are supplied (Smith *et al.*, 1980; Wood and Kelly, 1984).

Co-ordinate control of enzyme synthesis is believed to occur, with RuBisCO and PRK varying in a comparable manner under different conditions (Dijkhuizen and Harder, 1984). However, other evidence suggests that some independent variation in the synthesis of the two enzymes can occur (Leadbeater *et al.*, 1982).

Post-translational control is also believed to occur. An inactivated form of the RuBisCO enzyme showed faster mobility in polyacrylamide electrophoresis than an activated form (Jouanneau and Tabita, 1987). Rapid inactivation of RuBisCO can occur when cells were switched from limiting to non limiting inorganic carbon (C_i) concentrations, and in at least one case this was caused by proteolysis (Gottschal *et al.*, 1981). The nature of post-translational control is not known, but appears to be energy dependent (Tabita, 1988).

1.3.2: CO₂ uptake.

Because of the poor efficiency of RuBisCO at fixing CO₂, and the competition of O₂ as an alternative substrate, organisms require a much higher concentration of CO₂ at the site of RuBisCO within the cell than occurs in the external environment. At air saturation the effective K_m (CO₂) of cyanobacterial RuBisCO is about 600 μM, and aqueous media at 25 °C contains about 250 μM O₂ (Pierce and Omata, 1988). Intact

cells, however, have a much greater affinity for CO_2 than the isolated enzyme (Miller *et al.*, 1984). This higher affinity of whole cells indicates active accumulation of intracellular C_i (Miller *et al.*, 1988). C_i levels in excess of 1 000-fold over extracellular concentrations can be accumulated (Kaplan *et al.*, 1994). These elevated levels cannot be accounted for by simple diffusion across the membrane, indicating an active C_i transport system. (Kaplan *et al.*, 1991).

Most of the work on carbon concentrating mechanisms (CCMs) has been carried out using cyanobacteria or algae. Although similarities with acidophiles are likely, differences in at least the main C_i species present in the external environment exist. Cyanobacteria usually grow at pH values above 7.0, where the main dissolved C_i species is bicarbonate. Below pH 6.0, almost all dissolved C_i is in the form of CO_2 .

Cyanobacteria can actively transport both HCO_3^- and CO_2 (Miller, 1990). Electron transport inhibitors, uncouplers, and ATPase inhibitors all block C_i transport indicating that it is an energy dependent process (Badger and Andrews, 1982; Miller *et al.*, 1988; Spiller *et al.*, 1988). The transfer of cells from high C_i to low C_i results in the rapid induction of high affinity/high capacity CCM's (Coleman, 1991). However, cyanobacteria grown at enhanced CO_2 levels have a reduced ability to transport C_i , if placed in a low C_i environment (Kaplan *et al.*, 1980). CO_2 transport is constitutive in cyanobacteria, whilst HCO_3^- transport is inducible (Miller, 1990).

The actual mechanism of C_i transport and how this mechanism is coupled to energy is not known. Primary active transport may occur, or secondary transport could be driven by the movement of cations down an electrochemical gradient. Single transport systems for both HCO_3^- and CO_2 (Badger and Andrews, 1982), and separate systems for each species have been proposed (Miller, 1990). The main C_i form transported is not known, both HCO_3^- (Vолокита *et al.*, 1984), and CO_2 (Price and Badger, 1989a) have been implicated.

Little is known about the genes or gene products that are specifically involved in C_i transport. Most studies on high C_i requiring mutants, have resulted in organisms deficient in CO_2 fixation rather than CO_2 transport (Miller, 1990). The inactivation of a

gene cloned from *Synechococcus* appears to stop C_i transport, but the function of the gene product is not known (Ogawa, 1991).

In contrast to the photosynthetic bacteria little data on CCMs in chemolithotrophic bacteria is available. An inducible, high affinity CCM has been demonstrated in *Thiobacillus neapolitanus* (Holthuijzen *et al.*, 1987). This organism de-repressed RuBisCO synthesis three to five fold during autotrophic growth under air. It also responded to intracellular C_i limitation by the induction of a high affinity CCM. Active CO_2 transport required the presence of an exogenous energy source, and was dependent on the membrane potential. The C_i transport rate by this organism dropped drastically when the pH was increased from 6.0 to 7.5, suggesting that only CO_2 could be transported (at pH 6.0, 69% of C_i would be in the form of CO_2 , at pH 7.5 only 6.6% would be CO_2).

Thiobacillus versutus, unlike *T. neapolitanus*, requires levels of CO_2 above those in air for autotrophic growth on thiosulphate. Both organisms have similar levels and activity of RuBisCO, and so the lack of growth under air has been attributed to the absence of a high affinity CCM within *T. versutus* (Karagouni and Kelly, 1989).

1.3.3: Carboxysomes.

The high levels of CO_2 needed around the active site of RuBisCO results in another problem for the cells, namely how is a high CO_2 concentration retained without excessive energy loss across the cell membrane given the high lipid solubility of CO_2 ? The answer appears to be that the leakage of CO_2 from cells is much lower than predicted (Badger *et al.*, 1985). This indicates that there must be a barrier in the cell preventing CO_2 diffusion. Originally this barrier was thought to reside in the plasmalemma, but there is a physiological problem with this location. A barrier to CO_2 diffusion would also be a barrier to O_2 diffusion (Badger *et al.*, 1985). The restriction of O_2 diffusion in photosynthetic organisms such as cyanobacteria would result in toxic levels of O_2 building up (Badger *et al.*, 1985). Therefore, it has been suggested that the diffusion barrier resides within carboxysomes.

Carboxysomes are polyhedral inclusion bodies 100-200 nm in diameter (Codd, 1988). The number of carboxysomes increases in response to CO₂ limitation (Badger and Price, 1989b; McKay *et al.*, 1993). They have also been shown to contain RuBisCO (Codd and Marsden, 1984; Cannon *et al.*, 1991), and so are believed to be the main sites of CO₂ fixation within the cell. Several studies support the essential role of carboxysomes in CO₂ fixation. For example, inactivation of gene *ccmO* (an open reading frame flanking the operon encoding RuBisCO) in *Synechococcus* resulted in aberrant carboxysomes, and consequently a mutant that had a reduced ability to utilise the internal carbon pool (Marco *et al.*, 1994), and a *Synechocystis* mutant that did not produce carboxysomes required enhanced CO₂ levels for growth (Marcus *et al.*, 1992).

A model of carboxysome function was put forward in which the carboxysome shell was impermeable to both CO₂ and O₂ (Reinhold *et al.*, 1987). In this model bicarbonate is transported into the carboxysome where carbonic anhydrase (CA) converts it to CO₂, and then it is fixed by RuBisCO. Oxygen is prevented from entering the carboxysome and so cannot compete for the active site of RuBisCO. This model has been further modified (Reinhold *et al.*, 1991). It is believed that if the localisation of CA was at the centre of the carboxysome, and RuBisCO surrounded the CA, then an impermeable layer would not be needed at all. As the CO₂ was produced at the centre of the carboxysome by CA, it would diffuse to the surrounding RuBisCO and be fixed. High concentrations of RuBisCO within the carboxysomes would result in little CO₂ escaping into the cytoplasm.

Both these models rely on CA activity in the carboxysome. Other studies add some support to these models. RuBisCO in *T. neapolitanus* carboxysomes has been shown to be arranged in rows and concentric rings (Holthuijzen *et al.*, 1986). Secondly the expression of human CA within the cytoplasm of *Synechococcus* resulted in a high CO₂ requiring phenotype (Price and Badger, 1989b), suggesting that CA activity must reside in the carboxysome for efficient CO₂ utilisation. However, CA activity has never been unequivocally established in carboxysomes (English *et al.*, 1994). Also some

autotrophic thiobacilli do not appear to possess carboxysomes (Codd and Marsden, 1984), suggesting that alternative sites for CO₂ retention exist.

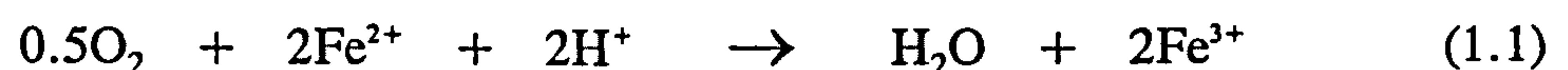
1.4: Metabolism of Chemolithotrophic Bacteria.

The chemolithotrophic bacteria of importance to bio-leaching oxidize ferrous iron, sulphur compounds, and minerals containing these two elements.

1.4.1: Iron oxidation.

Iron is one of the main elements in the earth's crust, but the role of living organisms in the global iron cycle is small compared to that in other cycles such as the nitrogen, carbon, and sulphur cycles (Nealson, 1983). The availability and suitability of iron for bacterial oxidation is limited by the low solubility, and rapid oxidation of iron at neutral pH. Only below pH 4 does iron solubility increase significantly, and the rate of spontaneous chemical oxidation decrease. The free enthalpy energy change of ferrous iron oxidation is low (-45.4 kJ/mol), making the cell yield from this energy source extremely poor (Boogerd *et al.*, 1989). Therefore, autotrophic life based on ferrous iron oxidation occurs primarily under acidic conditions, and large quantities of iron must be oxidized to satisfy the energy requirements for growth.

The bacterial oxidation of iron can be described by equation 1.1:



(Ingledew, 1982)

The rate of bacterial iron oxidation is affected by parameters such as temperature, and the concentrations of ferrous and ferric iron. High levels of ferrous iron can produce substrate inhibition, and ferric iron can produce both competitive and non competitive product inhibition (Jones and Kelly, 1983). Ferric iron inhibition is influenced by pH and by potassium iron concentration. Organisms also possess different affinities for ferrous and ferric iron. For example, *Leptospirillum ferrooxidans* possesses a higher apparent K_m for ferrous iron (0.25 mM) than *Thiobacillus ferrooxidans* (1.34

mM), and is less sensitive to ferric iron as an end-product inhibitor (Norris *et al.*, 1988).

Iron oxidation has only been extensively studied in *T. ferrooxidans*, and even here the mechanism has not been completely resolved (Kusano *et al.*, 1992). The iron-oxidizing system in *T. ferrooxidans* is believed to be a short chain based on three electron carriers (Kusano *et al.*, 1992). Ferrous iron is oxidized at the cell surface. Electron transfer then occurs, via rusticyanin, to the terminal cytochrome oxidase in the cytoplasmic membrane. A similar process probably occurs in all iron-oxidizing acidophiles with iron being oxidized in the acidic external environment, and the electrons generated being released to molecular oxygen within the cell, but the actual components of the electron transport chain may differ between taxonomically unrelated organisms.

1.4.2: Sulphur oxidation.

Most work on sulphur oxidation has concentrated on the non-acidophilic thiobacilli. Hydrogen sulphide, polysulphides, elemental sulphur, thiosulphates, polythionates, and sulphite can all be utilised by sulphur-oxidizing bacteria. Oxygen is normally used as the electron acceptor. At high substrate concentrations, such as those found in sulphur mines and sulphidic ores, significant amounts of sulphuric acid can be produced (Harrison, 1984). The following stoichiometry has been proposed for elemental sulphur oxidation (Suzuki and Takeuchi, 1992):



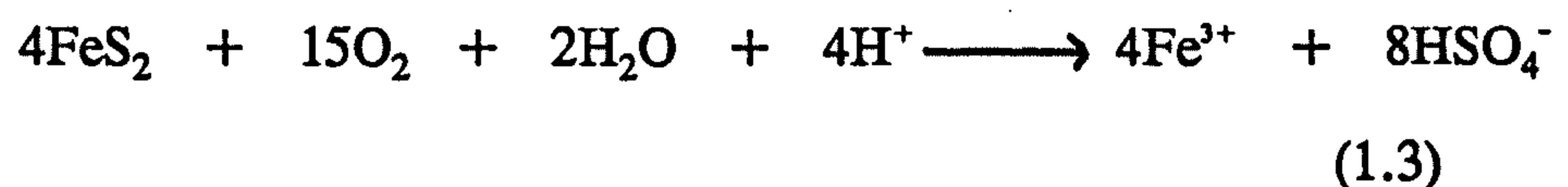
The actual oxidation pathway has not been completely resolved (Kelly, 1985; Pronk *et al.*, 1990). Enzymes catalysing the conversion of thiosulphate to tetrathionate have been identified, along with those that catalyse the final step of reduced sulphur oxidation, sulphite to sulphate.

As with iron oxidation systems, the overall sulphur oxidation process is likely to be similar in all acidophilic bacteria, but individual components may differ between taxonomically different groups.

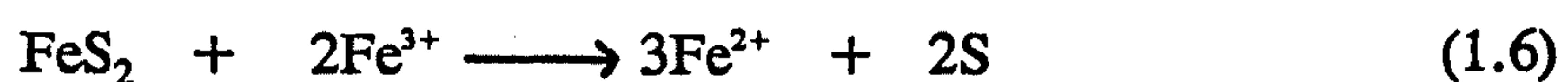
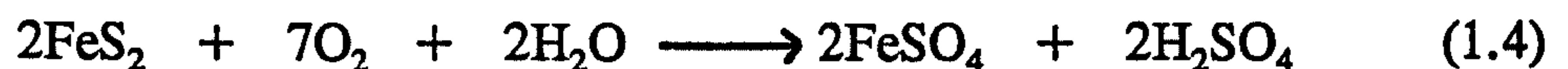
1.4.3: Oxidation of sulphide minerals.

Commercial interest in iron- and sulphur-oxidizing bacteria is related to their ability to oxidize a range of sulphide minerals. Natural metal leaching has been exploited since Roman times, and has been utilised in countries such as Canada, France, Peru, Spain, and the United States, but only in the past few decades has the involvement of micro-organisms been recognised (Lundgren and Silver, 1980). Bacteria that can oxidize both iron and sulphur are generally more active in the degradation of a range of minerals (Norris, 1989), although bacteria that oxidize only iron or sulphur probably remain important in the mixed cultures found both in nature and commercial operations. Maximum growth rates during mineral-oxidation may differ from those observed during iron-oxidation, because iron availability from the solid substrate can be limiting.

The oxidation of pyrite (FeS_2) can be expressed by equation 1.3 (Barrett *et al.*, 1993):



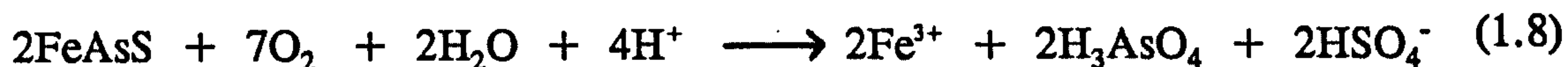
This overall reaction is thought to occur in the following four stages:



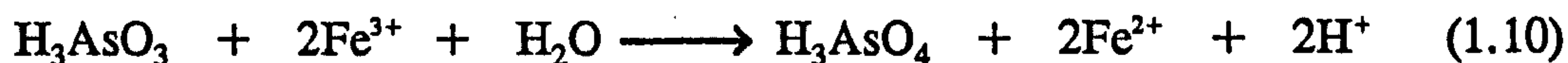
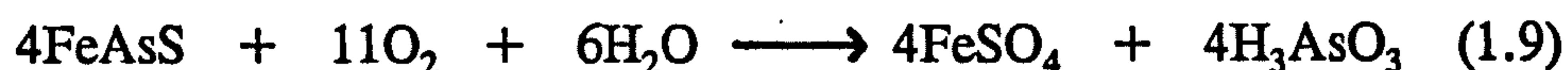
Reactions 1.4, 1.5, and 1.7 are bacterially catalysed. Reaction 1.6 is not bacterially catalysed but the ferric iron is produced bacterially. The involvement of bacteria is used to classify the type of oxidation observed. Direct bacterial oxidation refers to reactions such as 1.4, 1.5, and 1.7, where bacteria catalyse the reaction. Indirect bacterial oxidation is applied to chemical reactions where bacterial catalysis does not occur, but the oxidizing agent (usually Fe^{3+}) that produces the mineral dissolution is generated in a separate bacterially catalysed reaction (i.e. equation 1.6).

Reaction 1.6 can occur in the absence of cells and oxygen, but ultimately relies on bacterial regeneration of ferric iron from ferrous iron (Hutchins *et al.*, 1986).

Similar equations can be written for the bacterial oxidation of arsenopyrite, with the overall equation 1.8:



This reaction is believed to take place in two stages:



Reaction 1.9 is a result of direct bacterial oxidation, and 1.10 indirect bacterial oxidation.

The primary event of direct bacterial oxidation is probably the adsorption of the bacteria onto the mineral surface. This is believed to be a rapid process (Wakao *et al.*, 1984), but initial adsorption of cells is relatively weak, and the cells can be easily washed off the mineral. After several hours the bond between mineral and cell becomes more stable (Barrett *et al.*, 1993). *T. ferrooxidans* has been shown to selectively congregate on minerals at dislocations, grain boundaries and other discontinuities (Bagdigian and Myerson, 1986). Zones of corrosion are then formed at sites of long term adhesion of bacteria (Mustin *et al.*, 1992). The relative proportions of different bacterial types attached to minerals compared to those remaining free in suspension varies (Norris *et al.*, 1988). It is not known what affect (if any) this variation may have on overall mineral dissolution rates.

Other factors can affect the bacterial dissolution of minerals, including the nature of the mineral, the temperature, the pH, the oxidation-reduction potential, and the particle size (Lundgren and Silver, 1980). Generally the higher the temperature, the more rapid the dissolution. The optimum pH for bacterial oxidation of minerals is normally between 1.0-2.0. The greater the mineral surface area the greater the substrate availability, and thus the rate of mineral dissolution. Therefore, small particles will be more rapidly oxidized, but mineral grinding costs must be taken into consideration in preparation of minerals for commercial bioleaching.

1.5: Commercial application of bacterial oxidation.

As stated previously mineral leaching has been practised since at least Roman times (Lundgren and Silver, 1980). Dwindling mineral reserves, and pressures for a cleaner environment, have resulted in the need to use lower grade ores, and to seek less polluting methods of obtaining metals. Interest in the use of biological oxidation processes has increased over the last 25 years, because bio-oxidation processes can offer economical, energy-efficient, pollution-free processes, that can be operated by people with the minimum of training (Nicholson and Oti-Atakorah, 1993).

Mineral ores that are suitable for bio-oxidation are sulphidic in nature. Some of these are refractory to conventional extraction processes. A common cause of refractoriness in the case of gold is its encapsulation within the mineral sulphide (Hutchins *et al.*, 1986). These ores can be processed by roasting at temperatures of 600-800 °C to release metals. During roasting atmospheric oxygen is the oxidant, the sulphur content of the ore is oxidized to gaseous sulphur dioxide, arsenic, where present, to arsenous oxide, and the iron content to ferric oxide. Arsenous oxide must be contained and carefully disposed of, and stricter controls on the release of sulphur dioxide to the atmosphere have been gradually imposed. These two factors are major economic disadvantages to the use of roasting.

Alternative methods of mineral pre-treatment are known. Various chemical oxidation methods exist, including pressure, chlorine, nitric acid, and sulphuric acid oxidation (Barrett *et al.*, 1993). Only pressure oxidation has so far been proven in a full-scale commercial plant operation (Mason, 1992). Pressures of up to 20 atmospheres, at 200 °C drive the oxidation of the mineral. Atmospheric pollutants are not produced, instead liquid effluents containing sulphuric acid, arsenic acid, and ferric iron are produced.

Bacterial oxidation is an alternative to pressure oxidation. The process is essentially similar to pressure oxidation, with atmospheric oxygen as the main oxidant, and similar liquid effluent produced, but the process occurs at lower temperatures and

pressures. The main advantage of bio-oxidation over pressure oxidation is probably the lower standard of plant design and materials required (Nicholson and Oti-Atakorah, 1993). The choice between pressure oxidation and bio-oxidation, however, will depend on economics dictated by the specific circumstances, nature and treatability of different ores.

As well as the treatment of refractory ores, bioleaching offers an economic way of treating low grade ores and mining wastes. Copper can generally be produced from mining wastes using bio-oxidation at between a third to a half the cost of treating the mineral by conventional smelting processes (Torma, 1993).

1.5.1: Methods employed for bacterial oxidation.

There are five main methods by which bacterial oxidation has been applied (Barrett *et al.*, 1993). The method adopted is usually determined by the metal content of the ore, and the unit price of the metal to be recovered. Another factor to consider is whether a concentrate of the ore can be produced. This eliminates some of the unwanted gangue material, and can substantially reduce the amount of material to be treated.

a. Stirred tank reactors.

This system offers the most control over process parameters, enabling oxidation rates to be maximised. It is the most expensive method, and so is generally limited to precious metal extraction, as these have the highest unit price. With the possible exception of gold ores, only mineral concentrates are likely to be treated in this way. Finely ground mineral slurries are usually treated. A continuous culture system is normally employed with several reactors in series. Residence time in each reactor, the temperature and pH of operation, and mineral oxidation levels can all be carefully monitored and controlled. Capital costs are greatest with this system, because the equipment and instrumentation are relatively specialised compared to alternative bio-oxidation processes. Operating costs are also highest using this method mainly because

of the power requirement for agitation. Low value metals may be economically extracted in this way only as a secondary product to the precious metal.

b. Heap leaching.

The ore is placed within a heap, on an impermeable bed, that is specifically designed to optimise bacterial leaching. Lined concrete or PVC sheeting can be used to produce the impervious base. Rock size usually ranges from 50 cm to millimetres in diameter; with a good range in size necessary to allow liquid and gas penetration to all parts of the heap. Acidic leach solution is sprayed onto the top of the heap, and percolates through the ore body. The solution is collected in drainage dikes at the base of the heap. The metal is then extracted from solution, and the liquor re-cycled to the heap. Heaps may be as high as 200 m, and contain 50 000 to 300 000 tons of ore. It is a less expensive method than stirred tank leaching. Low grade ores, or metals with relatively low unit price can be treated in this way. The only control that can be maintained is over the percolating liquid added to the heap, and the process is slower than stirred tank leaching.

c. Dump leaching.

This is essentially identical to heap leaching, but here the ore is usually deposited without any considerations for bacterial oxidation, and is carried out on a much larger scale. Waste and ore below the metal grade that is economical to treat by heap or stirred tank leaching is dumped. Dumps can include rock, and waste tailings from previous metal extraction processes. The metal and sulphide mineral content of a dump is often low. Bacterial oxidation can be deliberately utilised to obtain the metal content, but unwanted oxidation can also occur in dumps, with the consequent production of acid drainage. Methods of controlling or preventing this unwanted bacterial oxidation are necessary to reduce environmental pollution.

d. Vat leaching.

Ore is placed within a large vat, and leach solution (or acid mine drainage) is allowed to percolate up through the pile of material as the vat is flooded. Modern vats are constructed by building a wall, and lining the dammed area with an impervious layer. The leaching solution is added at one end and collected at the other for re-cycling. The vat is periodically drained to allow gas diffusion. A substantial amount of water is required to flood the bed, and so this method can only be used in areas with an adequate water supply. Vat leaching operates on a smaller scale than heap or dump leaching, and has to date only been used for copper solubilisation.

e. *In situ* leaching.

When the metal content of an ore is too low to justify conventional mining *in situ* leaching may be possible. Cracks and fissures (natural or produced by blasting) are used to allow leach solution and gas diffusion through the ore body. *In situ* leaching is only possible where the desired metal is solubilised, and the ore body is surrounded by an impermeable rock layer. These constraints limit applications of *in situ* leaching. The most suitable sites are old mine workings, where the shafts and vents can be used for liquid and gas penetration. Leaching solution can be added either by piping or by injection wells. The solution flows through the ore and is collected in a series of wells in the impermeable rock below the ore body. The threat of metal rich acidic solution escaping into the ground water is a further potential environmental hazard. Despite limited applications and the potential problems, *in-situ* leaching has been used for both uranium and copper extraction.

1.5.2: Potentially extractable metals.

Table 1.1 lists the world production of some metals. This table gives an indication of the potential bacterial leaching could offer to the metal industry. Clearly the two most important metals based on their annual values are gold and copper.

Although other metals are extractable using bio-oxidation, only gold and copper will be considered further here.

Metal	Production Kilotonnes/year	Price \$/tonne	Total value Million \$/year
gold	1.73	1.163×10^7	20192
silver	11.4	1.31×10^5	1497
cobalt	20.9	2.46×10^4	513
uranium	28.6	2.08×10^4	593
nickel	930	7383	6863
molybdenum	108	6000	648
tin	158	5530	874
copper	7283	2338	17024
antimony	107	1650	176
zinc	5400	1003	5400
lead	2319	510	1180

Table 1.1: World production, prices and annual values of some metals. Table reproduced from Barrett *et al* (1993). Figures based on prices in 1990, and do not include information from the ex-communist countries.

Gold.

Gold exists in nature mainly as the native metal. It is estimated that 15-30% of the world's gold is contained within refractory ores (Barrett *et al.*, 1993). The most important gold encapsulating minerals are arsenopyrite and pyrite. Gold is normally recovered by direct leaching with cyanide to form soluble cyanide-gold complexes. Mineral encapsulation reduces gold recovery by preventing the aqueous cyanide ions from reaching the gold. Bacterial oxidation of the minerals exposes the gold, allowing conventional cyanidation to proceed. Bio-oxidation is, therefore, a pre-treatment stage to gold extraction.

The oxidation reactions of pyrite and arsenopyrite were given in section 1.4.3. Arsenopyrite is generally more susceptible to bacterial oxidation than pyrite. The extent of oxidation of the mineral required to achieve high gold recovery (>90%) depends on the distribution of gold within the mineral. When a single mineral type is present gold recovery is usually linearly related to total mineral oxidation (Lindström *et al.*, 1992), with a consequently high proportion of mineral needing to be oxidized. When the gold is encapsulated in a mixed mineral, i.e. pyrite/arsenopyrite, less total oxidation may be required, if the gold is preferentially associated with the arsenopyrite.

Small scale experiments have been carried out on a number of mineral concentrates. A Chilean flotation concentrate gave only 13% gold recovery by cyanidation prior to bio-oxidation (Maturana *et al.*, 1993). After bio-oxidation of this concentrate at 10% (w/v) solids in a 2 litre stirred reactor for 3 days, 95% gold was recovered. In comparison the same concentrate acid leached for 13 days only produced 34% gold recovery, clearly showing the role bio-oxidation had played.

A refractory gold concentrate from Greece was treated in continuous reactors consisting of a 40 litre feed tank and three cascade reactors of 10, 5 and 5 litres (Liu *et al.*, 1993). Prior to bio-oxidation gold recovery was only 16%. Recovery was improved by bacterial oxidation, but most of the mineral sulphide had to be oxidized to achieve good recovery levels, because the gold was associated with both pyrite and arsenopyrite.

Waste tailings from a New Guinea gold mine were treated in continuous and semi-continuous reactors (Hoffmann *et al.*, 1993). Continuous leaching used a 500 litre feed tank, three 400 litre reactors and a residue collection tank. Oxidation of 50% pyrite increased subsequent gold recovery from 49% to 78%, and reduced cyanide consumption.

Several pilot- and commercial-scale plants have been used to process refractory gold ores using bio-oxidation pre-treatment. Genmin, the mining division of the General Mining Union Corporation (Gencor), South Africa, have been operating a bacterial treatment process since 1984 (van Aswegen *et al.*, 1991). They began research into the

use of bacterial oxidation of refractory gold ores in the late 1970's. In 1984 a pilot scale plant was opened at the Fairview mine in South Africa to treat 750 kg of concentrate per day. 97% gold recovery was achieved in a residence time of 4 days. In 1986 a larger pilot-plant was set up capable of treating 10 tons of concentrate per day. This was about 40% of the mine's total concentrate production. A full scale plant was commissioned in 1991 to treat all the concentrate produced, and the original roasters were decommissioned.

Since the establishment of the Fairview treatment plant, the Genmin BIOX® bacterial oxidation technology has been used to commission four other plants (van Aswegen, 1993). In Sao Bento, Brazil, concentrate treatment capacity was increased by 12.5% using a bio-oxidation module capable of treating 120 tons per day. This plant was commissioned in 1990. The third plant using Genmin technology was commissioned at the Harbour Lights Mine in Australia during 1991, and has been operating at 40 tons per day since 1992. Asarco Australia Limited commissioned their bio-oxidation plant in 1992. It was designed to operate at 115 tonnes per day (Odd *et al.*, 1993). The largest plant to date has been commissioned by the Ashanti Goldfields Corporation Limited, Ghana (Nicholson and Oti-Atakorah, 1993). This plant is designed to deal with 760 tons of concentrate per day, and has been operating since March 1994.

Other companies utilising bio-oxidation processes include a Canadian company that has developed a mobile plant (Chapman *et al.*, 1993). It has been successfully used to treat a pyrite/arsenopyrite concentrate at 562 kg per day. Gold recovery was increased from 5% to 90%. The Equity Silver Mine, British Columbia, Bureau de Recherches Géologiques et Minières (BRGM), France, and Transworld Mining and Minerals (TWMM), Australia have all developed pilot- or commercial- scale plants (Lindström *et al.*, 1992).

The number of plants being commissioned indicates that bio-oxidation is an economically favourable process. Some of the reasons for choosing bio-oxidation over pressure oxidation were given for the Ashanti mine (Nicholson and Oti-Atakorah, 1993). These reasons included, significantly lower capital costs for biological oxidation,

biological oxidation is easy to operate making staff training easier and quicker, and further expansion on site is relatively straight forward.

Table 1.2 shows a comparison of the estimated capital expenditure and operating costs for three pre-treatment methods at the Sao Bento site in Brazil. Both the operating and capital costs are lower for the BIOX[®] treatment process (van Aswegen, 1993).

Pre-treatment Method	Capital Expenditure	Operating Costs
BIOX [®]	1.00	1.00
Roasting	1.92	1.11
Pressure oxidation	2.38	1.14

Table 1.2: Cost comparison of pre-treatment plants at Sao Bento, Brazil. Reproduced from van Aswegen (1993).

As well as the large number of stirred tank processes being developed heap bio-oxidation of gold ores is also being used (Brierley and Luinstra, 1993). The Newmont Gold Company have conducted laboratory tests using 45 kg columns. The results from these columns are to be used to develop a full scale process for refractory gold heap leaching in Nevada. Small test heaps (up to 27 000 tonnes) have already been set up, and full scale heaps are now planned.

Copper

It is estimated that 25% of the total copper production in the south west United States (Torma, 1993), and 15% of the world production (Barrett *et al.*, 1993) is produced using bioleaching. The main copper minerals are chalcopyrite, covellite, chalcocite, bornite, and enargite. Sulphidic copper minerals are conventionally treated by flotation and roasting, but all copper producers use an integrated bio-assisted leaching process in conjunction with their conventional methods (Torma, 1993). Stirred

reactor bio-oxidation of copper concentrates is unlikely to be economical, but heap, dump, vat and *in-situ* leaching have all been successfully used. The copper is solubilised during oxidation, and is removed from solution by cementation with scrap iron, or by solvent extraction coupled with electrowinning. Cementation was the predominant recovery method in the 1960's and 70's, but since then the solvent/electrowinning method has been favoured (Torma, 1993). Both direct and indirect leaching play a role in copper solubilisation.

1.5.3: Thermophiles in bio-oxidation.

Some consideration has been given to the use of thermophilic organisms in bacterial oxidation processes, because they offer several potential advantages over the traditionally employed mesophiles (Brierley, 1993):

1. Mineral oxidation is an exothermic reaction. Cooling is often needed in bio-oxidation processes to maintain the temperature at a level suitable for mesophile growth. The use of thermophiles would decrease cooling costs, and so overall process operating and capital costs would be reduced.
2. Reaction kinetics are faster at higher temperatures. Increased oxidation rates would reduce residence times in reactors, making processes more cost effective.
3. Thermophiles, and in particular extreme thermophiles, appear more effective at oxidizing refractory ores than mesophiles.

The use of thermophiles in bench- and pilot-scale bio-oxidation processes has been reported, but few, if any, have progressed further. A moderately thermophilic culture was tested for over 7 months in a 500 litre pilot plant for refractory gold concentrate pre-treatment (Spencer *et al.*, 1989). The moderately thermophilic culture had a wider operating temperature than the mesophile *T. ferrooxidans*, giving optimum oxidation rates in the range 30-49 °C. These rates were maintained up to a pulp density of 20% (w/v) solids. Gold recoveries of 75-80% were obtained upon cyanidation.

A direct comparison of a mesophilic culture grown at 35 °C and a moderately thermophilic culture grown at 45 °C has been carried out (Liu *et al.*, 1993). A refractory gold concentrate was used in a continuous system consisting of three reactors in series with a total volume of 20 litres. Almost complete mineral oxidation was required to give maximum gold recovery. The moderately thermophilic culture gave a higher level of total mineral oxidation, but suffered from selective loss of gold in the large quantities of foam produced by this culture. The conclusion of this study was that moderate thermophiles appear to offer an advantage over mesophiles if high mineral oxidation levels are needed, but excessive foaming would have to be controlled in any commercial application.

In many areas where bio-oxidation processes could be used, the water quality is low. Tolerance of bacteria to high salt concentrations would be a distinct advantage in these situations. A moderately thermophilic culture has been reported that can withstand 20 g/l NaCl (Budden and Spencer, 1993). Oxidation rates in continuous culture were not greatly affected by these salt levels, and the culture could operate at temperatures up to 55 °C. The culture has been tested on a number of concentrates, and consistently produces greater than 90% gold recovery.

Comparison of *T. ferrooxidans*, a moderate thermophile, and the extreme thermophile *Sulfolobus sp.*, in shake flasks and stirred tank reactors, indicated that the moderate thermophile was capable of improving gold recovery from a refractory gold concentrate from 0% prior to pre-treatment to 74% after pre-treatment at 50 °C (Hutchins *et al.*, 1987). Gold recovery from a second concentrate was improved the most by bio-oxidation with *Sulfolobus*, from 5.5% (before pre-treatment) to 91%. Despite the moderate thermophile oxidizing a greater proportion of this second mineral than the mesophile, both cultures gave similar gold recoveries about 55%.

A comparison of *T. ferrooxidans* and a *Sulfolobus* species in shake flasks at pulp densities from 2% (w/v) to 20% (w/v) indicated that the mesophile produced better oxidation rates at the high pulp densities (Lawrence and Marchant, 1988). However,

economic analysis indicated that *Sulfolobus* had the greatest potential, but improved oxidation at higher pulp densities would be required for commercial application.

The ability of several *Sulfolobus* strains and *Acidianus brierleyi* to oxidize a pyrite sample were tested in shake flask and airlift reactors (Larsson *et al.*, 1990). All tests were carried out at 2% (w/v) solids. Only *A. brierleyi* grew on the mineral tested, but this is believed to be as a result of only heterotrophic *Sulfolobus* species being tested!

A semi-continuous bench scale system for the pre-treatment of a pyrite/arsenopyrite concentrate at 70 °C with *Sulfolobus acidocaldarius* failed to achieve oxidation above a solid concentration of 1.5% (w/v) (Lindström and Gunneriusson, 1990). This failure of the culture to grow above 1.5% (w/v) solids was attributed to toxic levels of arsenic in solution.

T. ferrooxidans and an extreme thermophile were directly compared on a copper concentrate and a gold bearing arsenopyrite concentrate (Duarte *et al.*, 1993). Shake flasks, stirred tank reactors (up to 5 litre), and air lift reactors were all used during this study. The extreme thermophile failed to grow on the arsenopyrite concentrate in any of the systems employed. Again this was attributed to toxic levels of arsenic in solution. The extreme thermophile produced much better copper dissolution (80%) from the copper concentrate than *T. ferrooxidans* (40%). This study also indicated that the extreme thermophile performed better in the airlift reactors than in stirred tanks, probably a result of the lower shear stress conditions within the airlift reactor. Comparison of *Sulfolobus* BC and *T. ferrooxidans* on another copper concentrate produced similar results (Escobar *et al.*, 1993). *Sulfolobus* BC gave 85-90% copper recovery after treatment at 70 °C, whereas *T. ferrooxidans* only produced 50% recovery. These tests were only performed at 0.5% (w/v) solids.

The existing studies with moderate thermophiles indicate that they have similar bio-oxidation abilities to *T. ferrooxidans*, but can operate over a wider range of temperature. The conditions of application for moderate thermophiles is also very

similar to that currently employed for mesophiles. Therefore, the application of moderate thermophiles to situations where increased temperature operation is economically feasible, would appear to be a logical step.

Furthermore, moderate thermophiles and mesophiles are likely to co-exist in many situations, as the open nature of current bio-oxidation processes could not preclude one group or the other. All the Genmin BIOX® plants operate at temperatures between 40-45 °C (van Aswegen, 1993). This is a temperature at the limit of mesophile growth but at which moderate thermophiles are very active. The analysis of microbial populations from continuous bioleaching reactors operated at 35-40 °C in Australia, indicated that the culture was dominated by three organisms, one of which was a moderate thermophile (Goebel and Stackebrandt, 1994). If a reactor operated at this temperature has a moderate thermophile as one of the dominant organisms, then analysis of bioleaching reactors operated at 40-45 °C are very likely to indicate the presence of moderate thermophiles.

In comparison, the application of extreme thermophiles to commercial bio-oxidation is probably still some time off. Several important factors need to be addressed, with the lack of consistent results at high pulp densities, and the apparent low tolerance to toxic heavy metals such as arsenic, being two of the main limitations at present.

1.6: Project aims.

The overall aim of this work was to define the constituent organisms and interactions in moderately thermophilic cultures that were most effective in processing mineral sulphides in bioreactors. "Most effective" in this context means high rates of mineral oxidation, adequate resistance to relevant metal ions, and efficient carbon dioxide utilisation capacity.

To achieve these aims there were three main areas of work:

1. Characterisation and classification of moderate thermophile strains.

The selection of the most appropriate culture for a particular process is dependent on the characterisation of the constituent organisms within that culture. Classification of the main moderately thermophilic types would also eliminate any confusion over what organism or group of organisms was being used in each particular process.

2. Investigation of a moderately thermophilic culture capable of efficient CO₂ utilisation from air. A culture with this characteristic, not described for any other mineral sulphide-oxidizing moderate thermophiles, has been obtained. Characterisation of the key organisms within this culture would enable a definition of the interactions occurring. Comparison of this culture with the currently known poor CO₂ utilisers could also be undertaken.

3. Characterisation of growth and mineral oxidation of moderately thermophilic cultures on a range of mineral concentrates in bioreactors. Direct comparison with mesophilic and extremely thermophilic cultures would enable the advantages and disadvantages of moderate thermophiles to be assessed.

Chapter 2:

Materials and Methods.

2.1: Bacterial strains.

The moderately thermophilic iron-oxidizing bacteria used were all available within the Department of Biological Sciences at Warwick University. A list of the strains and their isolation sites is given in chapter 3. Key references to individual strains can be found as follows: *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko, 1978), TH1 (Brierley *et al*, 1978), TH3 (Brierley, 1978; Norris and Barr, 1985), ALV and BC1 (Marsh and Norris, 1983), THWX and YTF1 (Ghauri and Johnson, 1991), IC MIXED (Norris and Owen, 1993).

The mesophile *Thiobacillus ferrooxidans* DSM 583 and extreme thermophile *Sulfolobus* BC (Marsh *et al*, 1983) were used for comparative purposes in physiological studies.

2.2: Chemicals and growth media.

2.2.1: Chemicals.

Chemicals were obtained from the following manufacturers, unless otherwise stated:

British Drug House (BDH) Ltd., Poole, Dorset, England.

Fisons Scientific Equipment Ltd., Loughborough, England.

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Amersham International plc., Amersham, Buckinghamshire, England.

Distillers MG Ltd., Reigate, Surrey, England.

2.2.2: Media.

All media were sterilised by autoclaving at 121 °C for 15 min unless stated otherwise.

Salts medium:

	g/l
MgSO ₄ ·7H ₂ O	0.4
(NH ₄) ₂ SO ₄	0.2
KCl	0.1
K ₂ HPO ₄	0.1

Adjusted to appropriate pH with H₂SO₄.

Mineral leaching medium:

More growth occurs on sulphide minerals than on ferrous sulphate, and so the primary limiting nutrients were increased as indicated.

	g/l
MgSO ₄ ·7H ₂ O	0.5
(NH ₄) ₂ SO ₄	0.4
KCl	0.1
K ₂ HPO ₄	0.2

Adjusted to pH 2.0 with H₂SO₄.

Low phosphate medium:

As for salts medium, but one tenth the concentration of K₂HPO₄.

Solid Medium:

Low phosphate medium at pH 2.2, and either agarose type II or Gelrite solutions (in distilled water) were made and autoclaved separately. The salts solutions were allowed to cool to 50 °C, growth substrates were added, and then the salt solutions were mixed with the solidifying agent. Agarose was used at a final concentration of 0.5% (w/v), and Gelrite at 0.4% (w/v). Growth substrates added, at their final

concentrations within the media, were: ferrous iron (20 mM), yeast extract (0.02 % (w/v)), potassium tetrathionate (0.5 mM).

For pour plates, a 5 % (v/v) inoculum of mid-exponential cells was added to the salts solution prior to mixing with the solidifying agent. 25 ml of this medium was poured into each Petri dish.

2.2.3: Growth substrates and media supplements:

Ferrous sulphate.

1M stock solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were prepared. The pH was adjusted to 1.3-1.4 using H_2SO_4 , and the solution autoclaved at 115 °C for 10 min. For liquid media ferrous sulphate was added at a final concentration of 50 mM, and for solid media 20 mM.

Potassium tetrathionate.

100 mM stock solutions of $\text{K}_2\text{S}_4\text{O}_6$ (Fluka Chemicals Ltd., Gillingham, Dorset) were prepared, and autoclaved at 115 °C for 10 min. Potassium tetrathionate was used at a final concentration of 0.5 mM, as a reduced sulphur source during autotrophic growth of organisms unable to utilise sulphate.

Yeast extract.

Stock solutions of 1 % (w/v) yeast extract were prepared and sterilised by autoclaving. For both liquid and solid media, yeast extract was used at a final concentration of 0.02 % (w/v).

2.2.4: Minerals used during leaching studies.

Table 2.1 shows the mineralogical analysis for the three ore concentrates used in the leaching studies. The Mexican pyrite and the Bogosu arsenopyrite were obtained from Shell Research Ltd., whilst the Olympia concentrate originates from Greece. All minerals were ground to pass through a -200 mesh sieve.

MINERAL	ELEMENT (%)															
	P	S	Mo	Zn	Cd	Co	Ni	Mn	Fe	Cr	Mg	Al	V	Cu	Ca	As
BOGOSU ARSENOPYRITE	0.431	19.446	0.007	0.047	0.007	0.045	0.058	0.056	19.084	0.006	0.516	0.321	0.002	0.111	1.131	3.812
MEXICAN PYRITE	0.214	44.344	0.008	0.113	0.011	0.068	0.021	0.066	42.974	0.011	0.029	0.092	0.003	0.674	0.494	0.242
OLYMPIA ARSENOPYRITE	0.307	36.318	0.007	0.826	0.011	<0.01	<0.01	0.236	30.211	<0.01	0.067	0.055	<0.01	0.074	0.478	10.242

Table 2.1: Chemical analysis of mineral concentrates used in the leaching studies. The elements are shown as a percentage by weight of concentrate. Analysis of a digested mineral sample was performed by inductively coupled plasma mass spectrophotometry whilst at Shell Research Ltd..

2.3: Growth conditions.

For autotrophic growth on iron, the salts medium was adjusted to pH 1.7. Ferrous iron and 0.5 mM tetrathionate were added, and the cultures incubated under air, or gassed with 1 % (v/v) CO₂ in air.

For chemolithoheterotrophic growth on iron, 0.02 % (w/v) yeast extract was added instead of tetrathionate.

For heterotrophic growth, the salts medium was adjusted to pH 2.0, and 0.02 % (w/v) yeast extract and 10 mg/l ferrous sulphate were added.

For growth on minerals, the minerals were ground to pass through a -200 mesh sieve, and the appropriate amount added to the medium. For routine maintenance of cultures, 1 % (w/v) mineral was used. For autotrophic growth on minerals no further supplements were added, and the cultures were gassed with air or 1 % (v/v) CO₂ in air. For chemolithoheterotrophic growth on minerals, 0.02 % (w/v) yeast extract was added.

Cultures were routinely sub-cultured in 250 ml conical flasks containing 100 ml of medium. Gas mixes of CO₂ in air were supplied through sterile glass tubes (containing cotton wool filters) inserted through foam bungs. All moderate thermophile cultures were incubated at 45-48 °C in Gallenkamp orbital shakers at 100 rpm.

For growth of *T. ferrooxidans*, the salts medium was adjusted to pH 1.7, and 50 mM ferrous sulphate was added, with no reduced sulphur source. Cultures were incubated at 100 rpm and 30 °C.

Sulfolobus strain BC was grown on minerals at 68 °C, in pH 1.7 medium, in air-lift reactors.

For growth experiments (except mineral leaching studies), 1 l glass reactors fitted with water-jackets were used. Reactor temperature was controlled by circulating water baths (Conair Church Hill Ltd., Uxbridge, Middlesex). Air or 1% (v/v) CO₂ in air was delivered to the reactors at a flow rate of 400 ml/min, via sterile glass wool filters. Reactors were agitated using magnetic stirrers.

For mineral leaching work, 440 ml glass air-lift reactors fitted with water jackets were used. These reactors contained an inner glass draught tube. Gas was delivered to the bottom of the reactors through a central thin aeration tube. Reactors were gassed with air or 1% (v/v) CO₂ in air at a flow rate of 400 ml/min unless otherwise stated. A fresh 10% (v/v) inoculum, grown on the test mineral, was used for each experiment.

2.4: Growth assays.

Growth of the moderate thermophiles on iron was monitored by assaying ferrous iron in solution. Ferrous iron oxidation has previously been demonstrated as a valid method of growth estimation for these bacteria (Marsh and Norris, 1983). 1 ml culture aliquots (in triplicate) were titrated against 0.05 M ceric sulphate in 5% (v/v) H₂SO₄ with 1, 10-phenanthroline-ferrous sulphate complex solution as indicator, using a 50 ml digital burette (Brand, Germany).

For heterotrophic growth on yeast extract, culture optical density (440 nm) was used to follow growth.

During leaching studies both the pH and total iron in solution were measured to follow growth-associated, mineral bio-oxidation. Total iron was measured either by atomic absorption spectrophotometry using a Varian AA-1275 series atomic absorption

spectrophotometer, or by inductively coupled plasma mass spectrophotometry using a Jobin Yvon JY32 mass spectrophotometer.

Additional elemental analyses, including sulphur and arsenic measurements, were carried out using Jobin Yvon JY 32 and JY 38 mass spectrophotometers.

Cell protein was determined by the method of Lowry *et al.* (1951). Triplicate samples of between 10 ml and 100 ml were filtered through 0.2 μ m nitrocellulose filters (Sartorius-AG, Göttingen, Germany). Identical filters were also included with the protein standards to allow for an observed "quenching" effect of the protein by the filters.

2.5: Cell harvesting and lysis.

Cultures were harvested by centrifugation at 16 000 x g for 15 min, or 27 000 x g for 10 min in a fixed angle Beckman JA10 rotor or Beckman JA20 rotor, at room temperature. Following harvesting, cells were washed several times in fresh salts medium, pH 1.7, or acid water (pH 1.7). Washing steps included a series of slow spins at 1000 x g for 1 min to remove ferric iron precipitates, before final centrifugation.

If cells were to be lysed, they were first washed and re-suspended in 50 mM Tris, 10 mM EDTA (pH 8.5). One tenth volume of lysozyme (0.5 mg/ml) was then added and the cell suspension incubated at 37 °C until complete lysis had occurred (30-120 min).

2.6 Polyacrylamide gel electrophoresis (PAGE).

Denaturing PAGE was routinely carried out using 10% (w/v) acrylamide resolving gels according to the method of Laemmli (1970). Standard LKB (Bromma, Sweden) electrophoresis plates, a 2001 vertical electrophoresis unit, and an LKB 2197 power unit were used. Electrophoresis was performed at 40 mA per slab gel for 3-4 hours.

Protein standards (Pharmacia Inc. Uppsala, Sweden) used for SDS-PAGE were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya bean trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14 kDa).

Coomassie blue staining was routinely carried out overnight in:

Coomassie brilliant blue R-250	1 g
methanol	400 ml
glacial acetic acid	100 ml

Made up to 1 l with distilled water.

Gels were de-stained by frequent changes in the above solution minus Coomassie blue.

For silver staining, the method of Wray *et al.* (1981) was used.

2.7: Western blotting.

The method employed was based on that of Burnett (1981) with the following modifications.

Following electrophoresis the gel was allowed to equilibrate in blotting buffer (20 mM Tris (pH 8.0), 150 mM glycine, 20% (v/v) methanol, 0.1% SDS) for 30 min. A sandwich was then made for the blotter, consisting of a sponge sheet 1-2 cm thick, 3 thicknesses of Whatman 3MM paper, the gel, a sheet of nitrocellulose Hi-bond C, 3 more thicknesses of filter paper, and another sponge sheet. All layers were pre-wet in blotting buffer prior to assembly.

Blotting was carried out at 100 mA for 2.5-3 hours at 15-25 °C in a Gradipore electrophoresis electroblotting unit. Upon completion the filter was washed in distilled water to remove excess salt, and the gel stained using Coomassie blue stain. Proteins were visualised on the filter by washing in Ponceau S (0.5% (w/v) Ponceau S in 5% (w/v) trichloroacetic acid) for 10 min. Protein standards were marked with pencil, and

the Ponceau S completely removed using 500 ml TBS (50 mM Tris HCl (pH 8.0), 150 mM NaCl).

The filter was then blocked with 2% (w/v) milk powder in 20 ml TBS for 1-2 hours with gentle shaking. The TBS plus milk powder was poured off, and 20 ml fresh TBS plus milk powder containing the primary antibody added. This was left gently shaking overnight. Three 10 min washes in fresh TBS (20 ml) containing 0.1% Tween 20 were carried out. Fresh TBS plus 0.1% Tween 20 containing goat anti-rabbit peroxidase conjugant IgG was added, and incubated for 1-2 hours with gentle shaking. The secondary antibody was removed with two 10 min washes in excess TBS, and the filter stained with 100 ml staining solution for 5-20 min. The filter was then washed and stored in distilled water.

Staining solution:

Solution A:

1.5 g NaCl plus 1 ml 1M Tris HCl (pH 7.5) in 50 ml distilled water.

Solution B:

30 mg chloronaphthol in 10 ml methanol made up to 50 ml with distilled water.

50 μ l H₂O₂ was added to solution A, and then mixed with solution B.

This staining solution was used immediately.

2.8: Densitometer analysis and photography.

Stained gels were directly analysed on a computing densitometer (Molecular Dynamics, Sevenoaks, Kent). Stained gels and western blots were routinely photographed using a Pentax SP500 35 mm SLR camera with Agfa-Ortho film (ASA 25). Ilford contrast FF developer (Ilford Ltd., Mobberley, Cheshire), and Kodak universal liquid fixer (Kodak Ltd., UK) were used for developing and fixing both negatives and prints.

Percoll gradients were photographed using Kodak TMAX film (ASA 100).

2.9: Microscopy.

2.9.1: Phase contrast microscopy.

Cultures were routinely examined using an Unilux-11 stereoscopic microscope (Kyowa, Tokyo) at x 400 magnification. Photomicrographs were produced using a Leitz Dialux 22/22 EB microscope connected to a Leitz Vario Orthomat 2 automatic microscope camera (Leitz, W. Germany) using Kodak TMAX film (ASA 100).

For photomicrographs, washed cell suspensions were prepared, and 5 μ l of suspension was placed on to a cleaned microscope slide, which had been covered with 1% (w/v) Bacto agar.

2.9.2: Transmission electron microscopy.

A combination of methods, those of Kellenberger, Ryter and Sechaud (1958), Spurr (1969), and Wood and Kelly (1989), were used in sample preparation.

Harvested cells were fixed in 4% (v/v) glutaraldehyde in veronal acetate buffer (0.294 g/ml sodium veronal, 0.194 g/ml sodium acetate, 0.34 g/ml sodium chloride) for 2 hours at 4 °C. Cells were then post-fixed using 2% (w/v) OsO₄ in Kellenberger buffer (5% (v/v) veronal acetate buffer, 30 mM HCl, 10 mM NaCl) for 18 hours at 4 °C. Excess OsO₄ was removed by repeated washes in Kellenberger buffer.

Cells were embedded in 2% (w/v) Bacto agar, cut into 1 mm² blocks, and stained with 0.5% (w/v) uranyl acetate in Kellenberger buffer for 2 hours at 4 °C. Stained blocks were dehydrated through a graded ethanol series and embedded in Spurr resin (Agar Scientific Ltd., Stansted, Essex). Spurr resin was polymerised for a minimum of 8 hours at 70 °C.

Pre-sectioning was performed with a razor blade, to remove rough edges and excess resin. Sectioning was then performed using a diamond knife attached to a section cutter (Reichert, Austria). Sections were mounted on 3.05 mm OLD 200 or 400 copper grids (Agar Scientific Ltd., Stansted, Essex) and air dried for 30 min. Dried sections

were viewed using a Joel JEM-100S transmission electron microscope operated at 60-80 mA.

2.10: Percoll gradient centrifugation.

Percoll (Sigma Chemical Co.) gradients were prepared at concentrations between 50-100% using 0.2 M glycine buffer. Buffer pH was adjusted to between 2.0-2.9 using H₂SO₄. Higher concentration Percoll gradients required a lower pH buffer to maintain the gradient pH at 5.5 (the lowest stable value for Percoll). For a 71% Percoll gradient 0.2 M buffer at pH 2.6 was used.

8 ml of solution at the required Percoll concentration was placed in a Beckman JA21 tube. 200 μ l of washed cell suspension (OD₄₄₀ 2.0), in acid water at pH 2.0, was loaded on to each gradient. Gradients were centrifuged at 11 500 x g for 20-25 min at room temperature in a Beckman fixed angle JA21 rotor. Fractions down the gradient were extracted using a hypodermic needle, and examined by phase contrast microscopy. Percoll was removed from samples by diluting in buffer and centrifuging at 13 000 x g, to produce a Percoll free cell pellet.

2.11: Measurement of iron affinities.

A water-jacketed Rank oxygen electrode apparatus (Rank Bros. Ltd. Bottisham, UK) was used for all iron affinity determinations. The rate of oxygen consumption at a range of initial ferrous iron concentrations was used to determine iron oxidation rates. The standard reaction volume of 3 ml consisted of acid water pH 1.6, 50-200 μ l cell suspension, 150 μ l of appropriate ferrous iron solution (in acid water pH 1.6), and when ferric iron inhibition was measured, 133 μ l ferric iron solution, pH 1.6 (this gave an initial ferric iron concentration of 10 mM). All assays were carried out at 45 °C.

The amount of oxygen available within the reaction volume, at 45 °C, was estimated from tables of molecular oxygen solubility constants in water (Stephen and

Stephen, 1963). 191 $\mu\text{mol O}_2/\text{litre}$ was taken as 100% saturation. The zero was set by the addition of sodium dithionite.

Cells were harvested during each cultures phase of maximal iron oxidation, and washed several times in acid water (pH 1.6), with slow spins to remove ferric iron precipitates. All cell suspensions were re-suspended to the same optical density in acid water (pH 1.6). The cell suspension was kept on ice prior to additions to the electrode chamber. Initial assays were carried out to establish the volume of each cell suspension required to give appropriate rates of O_2 consumption. This volume varied between 50-200 μl , depending upon cell type and growth conditions (cell concentrations ranged from 20-50 μg protein per assay). Once the optimal volume was found, that volume of cell suspension was used throughout an experiment to minimise errors resulting from competitive inhibition of ferrous iron oxidation by cell concentrations (Suzuki *et al.*, 1989). Triplicate 200 μl samples were retained for protein assays.

Rates of oxidation were measured at ferrous iron concentrations of 1, 1.25, 1.66, 2, 5, 10, and 20 mM, in the presence and absence of 10 mM ferric iron. Experiments were initiated by the addition of the cell suspension, after the electrode containing the acid water, ferrous iron, and ferric iron had equilibrated at 45 °C, and 100% oxygen saturation. All assays were carried out in duplicate. The rate of oxidation of 5 mM ferrous iron was regularly measured throughout the day, to allow corrections to be made for any drop in activity of the cell suspension during storage on ice.

The data was analysed by three different graphical plots (see section 5.4.3) to obtain estimates of apparent Michaelis constants (K_m) for ferrous iron and inhibitor constants (K_i) for ferric iron.

2.12: Measurement of CO_2 uptake by whole cells.

Cells used during these assays were prepared in one of two different ways:

- i. "Growing cells" refers to cells that were taken from a culture during the phase of maximal iron oxidation, and assayed directly as described below.

ii. "Re-suspended cells" refers to cells that were taken from cultures during their phase of maximal iron oxidation, harvested by centrifugation, and re-suspended to an optical density (440 nm) of approximately 0.15. This cell suspension was then assayed as for "growing cells"

15 ml of cell suspension was added to a 25 ml quick-fit conical flask containing a magnetic stirrer. A suba-seal was used to seal the neck of the flask. The cell suspension was stripped of CO₂ by bubbling CO₂-free air through the suspension for 10 min at 45 °C. The flask was then clamped in a water bath above a heater-stirrer that maintained a constant temperature of 45 °C by means of a feed-back contact thermometer. The assay was initiated by the addition of the appropriate volume of 36 mM NaH¹⁴CO₃ (10.8 µCi/µmol).

At 1 min time intervals, duplicate 1 ml samples were removed through a long needle using a plastipak syringe (Becton-Dickinson, Dun Laoghaire, Co. Dublin). These samples were immediately filtered through 0.2 µm cellulose nitrate filters (Sartorius AG., W-3400, Göttingen, Germany) at a pressure of 10 bar, and then washed with 10 ml salts medium (pH 1.6). The filters were placed into 20 ml scintillation vials containing 200 µl absorption fluid (15 ml ethanolamine + 35 ml methoxyethanol). 10 ml Optiphase safe scintillant was then added, and scintillation counts performed using a LKB Wallac 1211 Minibeta liquid scintillation counter.

Triplicate 5 µl samples of 36 mM bicarbonate were retained for specific activity determination.

Protein determinations were made by the method of Lowry *et al* (1951). Triplicate 1 ml samples of re-suspended cells, and 50 ml culture volumes of growing cells were used.

When a regeneration period was used, assay flasks were incubated at 45 °C in a shaking water bath prior to assaying as above. 50 µl additions of 1M FeSO₄·7H₂O were made every 30 min for 3 hours.

As a result of the acidic media of the organisms studied, it was most appropriate to express the final assay concentrations of C_i used as a percentage (v/v) of CO_2 in air. The following calculations were therefore required:

1 mol of gas occupies 22.4 l at 25 °C, 1 atmosphere.

Using Charles law: $V_1/T_1 = V_2/T_2$.

Where V = volume.

T = temperature.

Therefore: 1 mol of gas will occupy 23.9 l at 45 °C, 1 atmosphere.

Since 1 μ l of 36 mM bicarbonate contains 36 nmol:

1 μ l 36 mM bicarbonate occupies 8.604×10^{-4} ml, at 45 °C.

Bicarbonate additions made were 15, 25, 50, 100, 200 μ l, and the assay vial volume was 37 ml.

These additions correspond to final CO_2 percentages (v/v) at 45 °C of:

0.04, 0.06, 0.12, 0.23, 0.47 respectively.

Bicarbonate specific activity was calculated from:

$$\text{Bicarbonate specific activity (cpm/nmol } CO_2) = \frac{(\text{cpm per } 5 \mu\text{l aliquot})}{(5 \times 36)}$$

Background was then subtracted.

The whole cell uptake rate (cpm/min) was calculated by plotting cpm (minus background) against time.

The whole cell specific uptake rate was calculated from the following equation:

$$\text{Specific Uptake Rate (nmol } CO_2/\text{min/mg protein)} = \frac{\text{whole cell uptake rate (cpm/min)}}{\text{Bicarbonate specific activity} \times (\text{mg protein/1 ml sample})}$$

2.13: Permeabilised whole cell assay for ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO).

The method used was based on that of Wood and Kelly (1989), and Leadbeater *et al* (1982).

Stock assay mixture (30 ml):

0.1 M Tris HCl (pH 8.0)	23 ml
0.25 M MgCl ₂	3 ml
reduced glutathione	19 mg
NaHCO ₃	112 mg
Na ₂ ¹⁴ CO ₃ (250 µCi)	125 µl
distilled water	3.85 ml

Harvested cells were washed in acid water (pH 2.4), then distilled water, and finally 0.1 M Tris HCl, pH 8.0, before being re-suspended to an optical density (440 nm) of 1.1 in 0.1 M Tris HCl. 3 ml of this suspension were then centrifuged per assay (giving approximately 1 mg/ml cells per assay). The cell pellet was re-suspended in 0.3 ml of 3% Triton-X-100, mixed briefly, and incubated for 15 min, at the normal, laboratory growth temperature of the organism being assayed.

0.9 ml of the bicarbonate mixture was added, mixed, and left for a further 10 min. 0.2 ml of ribulose-1,5-bisphosphate (RuBP) was added and mixed, to give 2 mM RuBP per assay (0.2 ml of water was used instead of RuBP as a control). Every 5 min for 30 min, 200 µl samples were taken, added to 100 µl 6 M phosphoric acid in a 20 ml scintillation vial and mixed. The vials were left open overnight in a fumehood to allow any unfixed CO₂ to escape. Then 10 ml of Optiphase safe was added to each vial, mixed well, and counts carried out immediately. Triplicate 10 µl samples of the bicarbonate mixture were added to 1 ml absorption reagent (15 ml ethanolamine, 35 ml methoxyethanol) and counted, to give the specific activity of the mix.

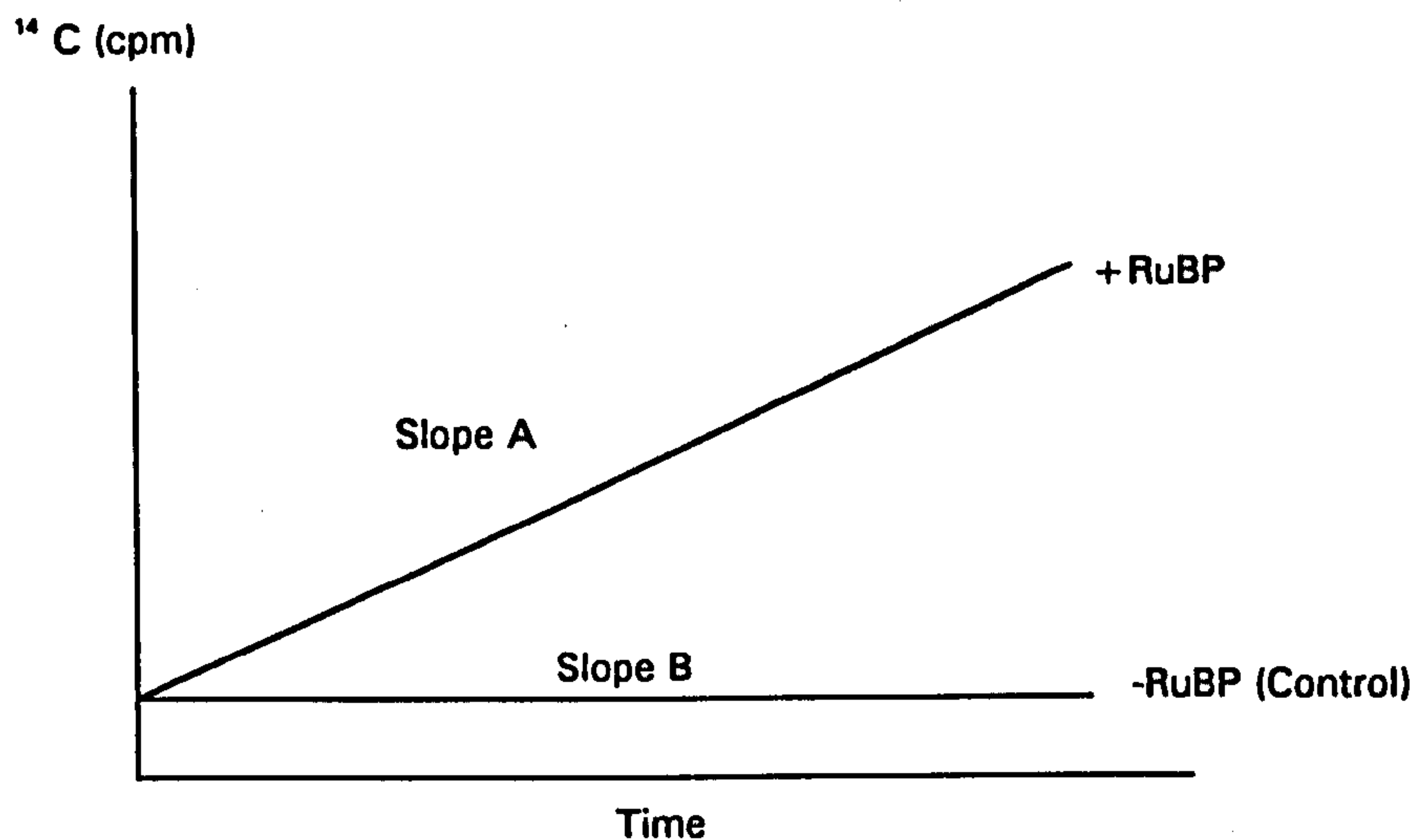
Triplicate 200 μl samples of each cell suspension were retained for dry weight assays.

The following calculations were used to estimate RuBisCO activity:

Stock bicarbonate mix contained 44 mM NaHCO_3 , therefore:

$$\frac{\text{Specific Activity (S.A.)}}{(\text{cpm/nmol CO}_2)} = \frac{(\text{cpm/10 } \mu\text{l}) \times 100}{44\,000 \text{ nmol}}$$

Background was deducted from all experimental counts, and then ^{14}C fixed was plotted against time.



$$\text{Enzyme activity (E.A.) (cpm/min)} = 7 \times (\text{slope A} - \text{slope B})$$

$$\frac{\text{Specific enzyme activity}}{(\text{nmol CO}_2 \text{ fixed/min/mg cells})} = \frac{\text{E.A.}}{\text{S.A.} \times \text{dry weight}}$$

2.14: DNA manipulation techniques.

Unless otherwise stated all methods were based on those of Maniatis, Fritsch, and Sambrook (1982).

2.14.1: Preparation of chromosomal DNA.

Nine litres of heterotrophically grown cells were harvested, washed three times in acid water pH 2.5, and then once in distilled water and once in 0.1 M Tris HCl pH 8.0. The cells were then washed in 0.12 M NaCl, 0.05 M EDTA pH 8.0, and re-pelleted. The pellet was re-suspended in 10 ml Tris-sucrose pH 8.0 (0.05 M Tris pH 8.0, 25% sucrose). 0.2 mg/ml lysozyme was added, and the tube incubated at 37 °C, until lysis was complete (10-60 min). 15 ml 0.25 M EDTA were added, and the tube incubated for a further 50 min at 37 °C. Sarkosyl was then added to a final concentration of 2% (v/v), and the tube incubated at 50 °C for 1 hour. Samples were then prepared for caesium chloride gradient centrifugation (Maniatis *et al.*, 1982). Centrifugation was performed in a Beckman L8 ultracentrifuge and Vti50 rotor at 15 °C, 45 000 x g for 18 hours.

After centrifugation the chromosomal band was extracted with an 18 gauge needle and 10 ml syringe, and the ethidium bromide removed with T.E. (10 mM Tris HCl, 1 mM EDTA, pH 8.0) saturated butan-1-ol (Maniatis *et al.*, 1982). Caesium chloride was removed by dialysis against two changes of 5 l of T.E.. DNA was then ethanol precipitated (Maniatis *et al.*, 1982), and re-suspended in 1 ml T.E..

2.14.2: Analysis of DNA base composition.

For DNA base composition analysis stock DNA was diluted in 8 ml of 0.1 x SSC (15 mM NaCl, 1.5 mM trisodium citrate) to give about 30 µg DNA/ml. Ultrapure DNA standards (Sigma Chemical Co. Ltd.) of *E. coli* strain B, and *Micrococcus luteus* were also dissolved in 0.1 x SSC to a final concentration of about 17 µg/ml. All samples and standards were dialysed for 48 hours against three changes of 5 l of 0.1 x SSC.

1.5 ml of each sample was then scanned using a HP 8452A UV-visible spectrophotometer connected to a HP 89090A Peltier temperature controller. DNA melting temperature was calculated using the DNA melt software package (Hewlett Packard Co., Germany).

2.14.3: DNA hybridisation studies.

Based on the method of Sharp and Williams (1988).

Labelled probe DNA was produced by J. Owen, a technician in the Department of Biological Sciences at Warwick University, using a nick translation kit (Amersham Int. plc., England), and a column of G50 fine Sephadex, for re-purification.

600 μ g DNA were added to 4 ml SSC in a 250 ml flask. 4 ml 1 M NaOH (pH 14) was added, mixed, and left for 20 min at room temperature. The flask was then placed on ice, and the liquid neutralised to pH 7.0-7.5 with acid buffer (1 part 1M HCl: 1 part 1 M Tris: 2 parts NaCl). Nitrocellulose filters, 5 cm in diameter and 0.2 μ m pore size (Sartorius AG., W-3400, Göttingen, Germany), were floated on 3 x SSC prior to binding DNA by filtering at 1 ml/min. Filters were rinsed with 20 ml cold 3 x SSC, and blotted to remove excess liquid from the backs of the filters. Filters were then placed between layers of filter paper in glass Petri dishes in a vacuum oven at room temperature overnight. DNA was baked on to the filters at 80 °C for 4 hours. The filter was cut in to 5 mm diameter discs, and stored in a vacuum desiccator.

The amount of DNA bound to the 5 mm filter discs was estimated using the Burton assay. 0.5 ml 0.5 N HClO₄ was added to each filter, and heated at 70 °C for 20 min. Once cool, 1 ml Burton reagent (100 ml glacial acetic acid, 1.5 ml H₂SO₄, 1.5 g diphenylamine, 0.5 ml acetaldehyde) was added, mixed, and incubated at 37 °C overnight in the dark. Optical densities (600 nm) were then read, and DNA concentrations estimated. Calf thymus DNA in 5 mM NaOH was used as a standard.

Pre-incubation of filters was carried out in 500 μ l Denhardt medium (0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl pyrrolidone, 0.02% (w/v) Bovine serum albumin fraction V, in 3 x SSC, pH 7.0), at 60 °C for 4 hours, with gentle shaking. The

hybridisation mixture was made by adding a sufficient quantity of labelled DNA to 3 x SSC/13% formamide, to give 200 μ l mixture per tube at 20 000 cpm/tube. This hybridisation mixture was boiled 3 x 10 min with rapid cooling on ice in between each boiling step. 200 μ l hybridisation mixture was aliquotted on to each filter, and incubated at 60 °C for 20 hours. The hybridisation solution was then removed whilst the tube was on ice. Filters were washed twice in 1 ml cold 3 x SSC, and blotted dry. Filters were then placed in scintillation vials and heated to 80 °C for 30 min in a vacuum oven. 3 ml Optiphase safe scintillant was added, and counts performed using a LKB Wallac 1211 Minibeta liquid scintillation counter.

2.14.4: PCR amplification of RuBisCO LSU gene fragment.

A fragment of the RuBisCO large subunit (LSU) gene, encoding amino acid residues 71-200 (Spinach enzyme numbering), was amplified from chromosomal DNA using the polymerase chain reaction technique (Saiki *et al.*, 1985). 43 μ l of reaction mixture (5.5 μ l 10 x pol. buffer, 1.65 μ l 50 mM MgCl₂, 4.4 μ l dNTP's, 0.5 μ l 1% w-1, 1.1 μ l bovine serum albumin, 34.15 μ l distilled water), 1 μ g template DNA and 500 ng of each primer were used per reaction. The reaction mixture was overlaid with 50 μ l of mineral oil, and denatured at 94 °C for 5 min prior to adding 2.5 U Taq DNA polymerase (GIBCO BRL Life Technologies Ltd., Scotland). Annealing took place at 50 °C for 1 min, followed by extension at 72 °C for 1 min. Thirty cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 5 min, were conducted using a Hybaid Combi thermal reactor TR2. Primers were based on those used by Holden and Brown (1993), and were synthesised using an Applied Biosystems DNA synthesiser.

Primer 1: 5'-ACC TGG ACC GTG GTG TGG-3' (encoding Thr-65 to Asp-70).

Primer 2: 5'-GTT GAT GTT CTC GTC GTC CTT-3' (encoding Lys-200 to Asn-206).

Amino acid positions are based on those of the spinach RuBisCO enzyme (Zurawski *et al.*, 1981).

After amplification, the PCR products were separated by electrophoresis on a 1.0% (w/v) agarose minigel (Maniatis *et al.*, 1982). PCR products were extracted from the gel and purified using a QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, USA). Products were then cloned using the TA cloning kit (Invitrogen Corp., San Diego, USA). Plasmid preparations were made using a QIAprep Spin Plasmid Kit (QIAGEN Inc., Chatsworth, USA), and inserts sequenced on an Applied Biosystems automatic sequencer model 373A. Primers used for sequencing were the RuBisCO LSU primers described, and the M13 Reverse Primer, and TA -44, a primer specific to a region 44 bases upstream of the inserted PCR product in the pCRTMII vector (Invitrogen Corp., San Diego, USA).

2.14.5: Estimation of DNA concentration.

Two methods were used, as described by Maniatis *et al.* (1982): the minigel method using Lambda DNA digested with *Hind* III as a standard, alternatively absorbance of the DNA solution at 260 nm was measured, and it was assumed that 50 µg/ml chromosomal DNA, and 33 µg/ml oligonucleotides had an absorbance of 1.0.

2.15: Whole-cell hybridisation with fluorescence-labelled 16S rRNA-targeted oligonucleotide probes.

Methods were based on those of Manz, *et al* (1992).

2.15.1: Probe design.

The partial 16S rRNA sequence data for moderate thermophile strains ALV, BC1, and TH3 (Lane *et al.*, 1992) was used to identify possible target regions for strain specific oligonucleotide probes. Three probes were designed to different regions of the 16S rRNA sequence specific for strain TH3. All three probes were rhodamine labelled at the 5' end. In addition the eubacterial probe Eub 338 (Amann *et al.*, 1990a)

labelled with rhodamine or fluorescein was used. All oligonucleotides were purchased from Severn Biotech Ltd., Kidderminster, Worcs.

The three specific probes were:

TH3 825 5' AAT GAG GCC CCA CAC CTA GT 3'

Coding for region 825-844 (*E. coli* numbering).

TH3 1166 5' ACC TTC CTC CGA ATT AAC 3'

Coding for region 1166-1184 (*E. coli* numbering).

TH3 1245 5' TTG GCG ACC CTT TGT ACC 3'

Coding for region 1245-1262 (*E. coli* numbering).

The eubacterial probe was:

Eub 338 5' GCU GCC UCC CGU AGG AGU 3'

Coding for region 338-355 (*E. coli* numbering).

Figure 2.1 shows the target sequences for each of the three specific probes, and the sequence alignment of 16S rRNA in the vicinity of the target sites for moderate thermophile strains ALV, BC1, and TH3, and for *E. coli* and *B. subtilis*.

a. TH3 825	ACU AGGUGUGGGG CCUCAUU
strain TH3	GGC... ..CGA
strain ALV	GAUG.GC.C. GGGGG..CCA
strain BC1	GGU...C.CC GGG .CCA
<i>E. coli</i>	UCG... U....GUU.U G.C.U.GAGG
<i>B. subtilis</i>	AGUG.. .A....UA.. GGGUU.CCGC

b. TH3 1166	GUUAAUUC GGAGGAAGGU
strain TH3	GA..... GGGG
strain ALV	GG.ACG.CC. GGGG
strain BC1	GT.AC..AC. GGGG
<i>E. coli</i>	GU.A...ACU GGGG
<i>B. subtilis</i>	GU.AC..AC. GGGG

c. TH3 1245	GGUACAAAG GGUCGCCAA
strain TH3	GGC C.....C CCG
strain ALV	GGC GCCG....C. ..C...G.CC UCG
strain BC1	GGU CCCG....C. ..A...G.CG GCG
<i>E. coli</i>	GGC GCA..... A.AA..G.CC UCG
<i>B. subtilis</i>	GGA CA.A..... ..CA..G..C CGC

Figure 2.1: Target sequences for strain TH3 16S rRNA specific oligonucleotide probes. The target sequence is displayed in the upper row, and sequence alignments for strains ALV, BC1, TH3, and for *E. coli* and *B. subtilis* are shown in the region of the target sequences. Dots indicate the presence of the same residue as found in the probe target sequence, a gap indicates a residue deletion. a. Target region of probe TH3 825, b. target region for probe TH3 1166, c. target region for probe 1245.

2.15.2: Cell fixation.

1 ml of a fresh, mid-exponential culture was taken for each cell type. Strains TH3 and NAL were grown heterotrophically on 0.02% (w/v) yeast extract, *Bacillus subtilis* and *E. coli* were grown in LB broth. The 1 ml samples were fixed in 3 ml of 4% paraformaldehyde in 0.2 M sodium phosphate (pH 7.2) for a minimum of 3 hours at 4 °C in the dark. 100 µl of fixed cell suspension was then added to 10 µl of 1% Triton, and centrifuged at 6000 x g for 1 min. The supernatant was discarded, and the cell pellet re-suspended in 10 µl 0.1% Triton. 3 µl of each cell suspension was spread onto prepared slides to an area of 5 mm². Cell smears were air dried for 1 hour at 37 °C, before being dehydrated through a graded ethanol series (50, 80, 96% (v/v) ethanol, 3-5 min in each).

2.15.3: Slide preparation.

Slide surfaces were cleaned by soaking overnight in ethanolic potassium hydroxide (10% (w/v) KOH). They were first rinsed with ethanol, and then thoroughly with double-distilled water, and air dried. Cleaned slides were coated with a gelatin solution (0.1% gelatin, 0.01% chromium potassium sulphate) at 70 °C. Slides were allowed to dry in a vertical position.

2.15.4: Whole cell hybridisations.

Hybridisation was carried out in a properly sealed moisture chamber to prevent evaporative loss of the hybridisation solution. A slip of Whatman 3 MM paper was soaked in a salt solution of the same osmolarity as the hybridisation buffer, and placed in the bottom of the chamber. The chamber was allowed to equilibrate at the appropriate hybridisation temperature, for at least 30 min prior to hybridisation. 1 µl of probe (approximately 40 µg/ml) was added to 8 µl of hybridisation buffer. Two hybridisation buffers were used:

1. 0.9 M NaCl, 0.1% (w/v) SDS, 20 mM Tris HCl (pH 7.2), 100 µg/ml polyA.
2. 0.9 M NaCl, 0.1% (w/v) SDS, 20 mM Tris HCl (pH 7.2), 20% formamide.

The cell smear was carefully covered with the hybridisation mix, and the slide placed in the chamber at the appropriate hybridisation temperature for 1.5 to 3 hours. The hybridisation mix was then washed off with 2 ml of pre-warmed wash solution (20 mM Tris.HCl (pH 7.2), 0.01% (w/v) SDS, 180 mM NaCl, 5 mM EDTA), and the slide washed for a further 20 min in 50 ml pre-warmed wash buffer. Slides were briefly rinsed in distilled water, to remove excess salt, and then air dried. Prior to viewing, 20 μ l of Citrifluor was placed on to each cell smear.

Slides were viewed under phase contrast and epifluorescence using a Zeiss Axioskop microscope fitted with filter sets 9 and 15.

Chapter 3:
Characterisation And Classification Of Moderately
Thermophilic Iron-Oxidizing Bacteria.

3.1: Introduction.

The existence of bacteria in ore deposits and thermal springs capable of growth at temperatures between 40-60 °C was demonstrated in 1977 (Brierley and Lockwood, 1977; Le Roux *et al.*, 1977; Golovacheva and Karavaiko, 1978). Since that date more moderately thermophilic, acidophilic bacteria have been discovered (Norris, 1989a). These moderately thermophilic bacteria can be separated into those capable of iron- and sulphur-oxidation and those capable of sulphur-oxidation only (Norris, 1989a). The iron-oxidizing bacteria are Gram-positive, and have optimal growth temperatures around 50 °C. Some of these bacteria have been classified as members of the new genus *Sulfobacillus*, whilst the others are still known only by their isolation codes.

The genus *Sulfobacillus* consists of facultatively thermophilic, autotrophic, acidophilic, spore-forming bacteria. Three isolates have so far been placed into this genus (see section 1.1.2). All three of these isolates originated from the Soviet Union.

Harrison (1986) carried out nucleic acid analysis on five of the unnamed moderately thermophilic isolates; strains ALV, BC1, TH1, TH3, and LM2. For the origins of these isolates see Table 3.1. The range in G+C content obtained (49.5-68.5 mol%) clearly indicated that these bacteria represented more than a single genus. Three of the isolates (strains ALV, BC1, TH3) were further characterised by comparison of partial 16S rRNA sequences (Lane *et al.*, 1992). All three isolates were placed close to the division between low and high G+C Gram positive bacteria, with strains ALV and BC1 within the low G+C sub-division, and strain TH3 firmly affiliated with the high G+C sub-division. Ghauri and Johnson (1991) studied three other moderately thermophilic isolates and compared them to strain TH1. Once again G+C contents of these isolates had a wide range from 43-68 mol%, but no further classification of isolates was made.

The aim of this work was to characterise and classify the moderately thermophilic iron-oxidizing isolates available within this laboratory. This would facilitate identification of isolates already being studied in other laboratories and that of

new isolates as they are obtained. Table 3.1 lists the moderately thermophilic iron-oxidizing bacteria available at the start of this project.

Isolate Name or Code	Isolation Site
<i>Sulfobacillus</i> <i>thermosulfidooxidans</i>	Nikolaev sulphidic ore dump, Eastern Kazakhstan.
Strain BC1	Coal pile, Birch Coppice colliery, Warwickshire, UK.
Strain N	Hot spring, Yellowstone National Park, USA.
Strain CLP1	Hot spring, Clear Lake, Yellowstone Natl. Park, USA.
Strain TH3	Chino copper mine dump, Hurley, New Mexico.
Strain YTF1	Frying Pan hot spring, Yellowstone Natl. Park, USA.
Strain THWX	Coal pile, Wrexham, UK.
Strain ALV Strain NAL	Coal spoil, Alvecote, Warwickshire, UK.
Strain LM1	Hot spring, Lake Myvam, Iceland.
Strain TH1	Hot spring, south-western Iceland.
Strain IC2A Strain IC2B Strain IC2C Strain IC3A Strain IC3B Strain IC3C Strain IC4	Hot spring complex, Iceland.

Table 3.1: Moderately thermophilic iron-oxidizing bacteria available within this laboratory.

Original isolation sites are given.

Previous work had indicated that several of the isolates were very closely related to one another; for example strains TH1 and BC1 were thought to be different isolates

of the same species (Norris and Barr, 1985). Further unpublished work, including the study of electrophoretic protein patterns, indicated that other isolates were identical or very closely related to one another. Strains TH1, BC1, LM1, IC3A, and IC3C were almost identical morphologically, physiologically and in electrophoretic patterns, and as such were considered to be isolates of the same organism. Strains IC2A, IC2B, IC2C, and IC3B were all identical, but differed from strain BC1. Finally strains N and CLP1 were found to be identical. With this identical nature of some of the isolates in mind strains ALV, BC1, IC2B, IC3B, IC3C, IC4, LM1, N, NAL, TH3, THWX, and YTF1 were selected for further characterisation.

3.2: Analysis of DNA:DNA hybridisation values.

DNA:DNA hybridisation is the conventional method used for delineation of species (Schleifer and Stackebrandt, 1983; Johnson, 1984). Filter hybridisation experiments were carried out with a range of moderately thermophilic iron-oxidizing bacteria, using DNA samples prepared by a technician (J. Owen) within this laboratory. Tritium-labelled probe-DNA was also prepared by J. Owen. Hybridisation was measured by taking the scintillation counts obtained in the homologous system (i.e. same probe and filter bound DNA) and expressing all other results as a percentage of this value.

Initial experiments produced some anomalous results. These anomalies were found to be caused by variable amounts of DNA binding to the filters prior to hybridisation. When the effect of filter-bound DNA concentration on hybridisation was considered it was found that at high concentrations (30-35 μg) the counts obtained remained constant, but at lower concentrations, the counts became progressively more proportional to the amount of DNA filter-bound (Figure 3.1). Consequently, the accuracy was too critically dependent on the concentration of DNA bound, if the amount of DNA varied between filters. Therefore, in all subsequent experiments the amount of DNA bound to the filters was maintained above 30 μg in order to eliminate this DNA concentration dependency.

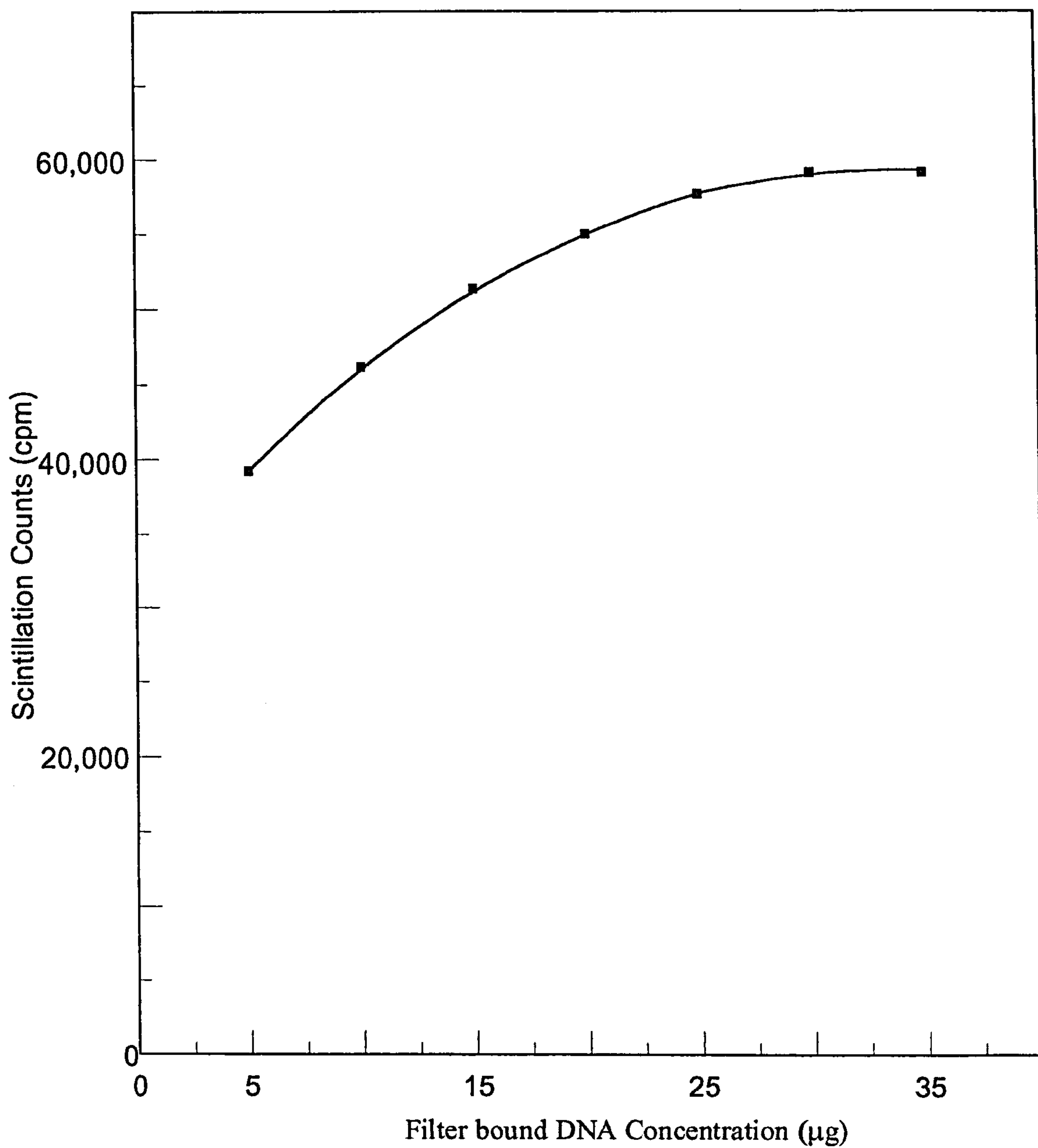


Figure 3.1: The effect of filter bound DNA concentration on hybridisation. Varying amounts of the stock DNA of *S. thermosulfidooxidans* was bound to filters and then hybridised with the labelled *S. thermosulfidooxidans* probe DNA.

After these initial experiments insufficient material remained to produce one large hybridisation matrix containing all DNA samples with all probe DNA. Instead three smaller matrices were used containing reference strains from each of the other matrices. This allowed comparison between all isolates without using large amounts of the available DNA, but resulted in some gaps in the overall results. Each of the three matrices were carried out in duplicate, and the average counts of triplicate readings were taken.

Table 3.2 is a summary of the results obtained from all three of the hybridisation matrices. Strain BC1 showed high hybridisation values to *S. thermosulfidooxidans*, the Russian type strain ($> 90\%$). These two isolates showed very low hybridisation values to any of the other strains. Isolate TH3 gave hybridisation values of $< 5\%$ to any of the other strains. The remaining four isolates ALV, NAL, 2B, and N showed hybridisation values to each other of $\geq 75\%$, and $< 15\%$ to strains TH3, BC1, and *S. thermosulfidooxidans*.

A software package (NTSYS-pc, Exeter Publishing Ltd.) was used to produce a dendrogram of the results by the UPGMA (unweighted pair group method using arithmetic averages) clustering technique (Figure 3.2). This dendrogram indicates that these iron-oxidizing bacteria fall into three distinct groups. *S. thermosulfidooxidans* and strain BC1 make up the first group. The second distinct group consists of the four isolates ALV, NAL, IC2B, and N. The third main branch to this dendrogram consists of a single isolate, strain TH3.

The close relationship between strains ALV and NAL was expected, as these two isolates were obtained from the same sample site several years apart, and appeared almost identical in most of the studies carried out. Strain ALV has an atypical morphology, however, growing autotrophically on ferrous iron as distorted filaments, while other members of the group are rod-shaped.

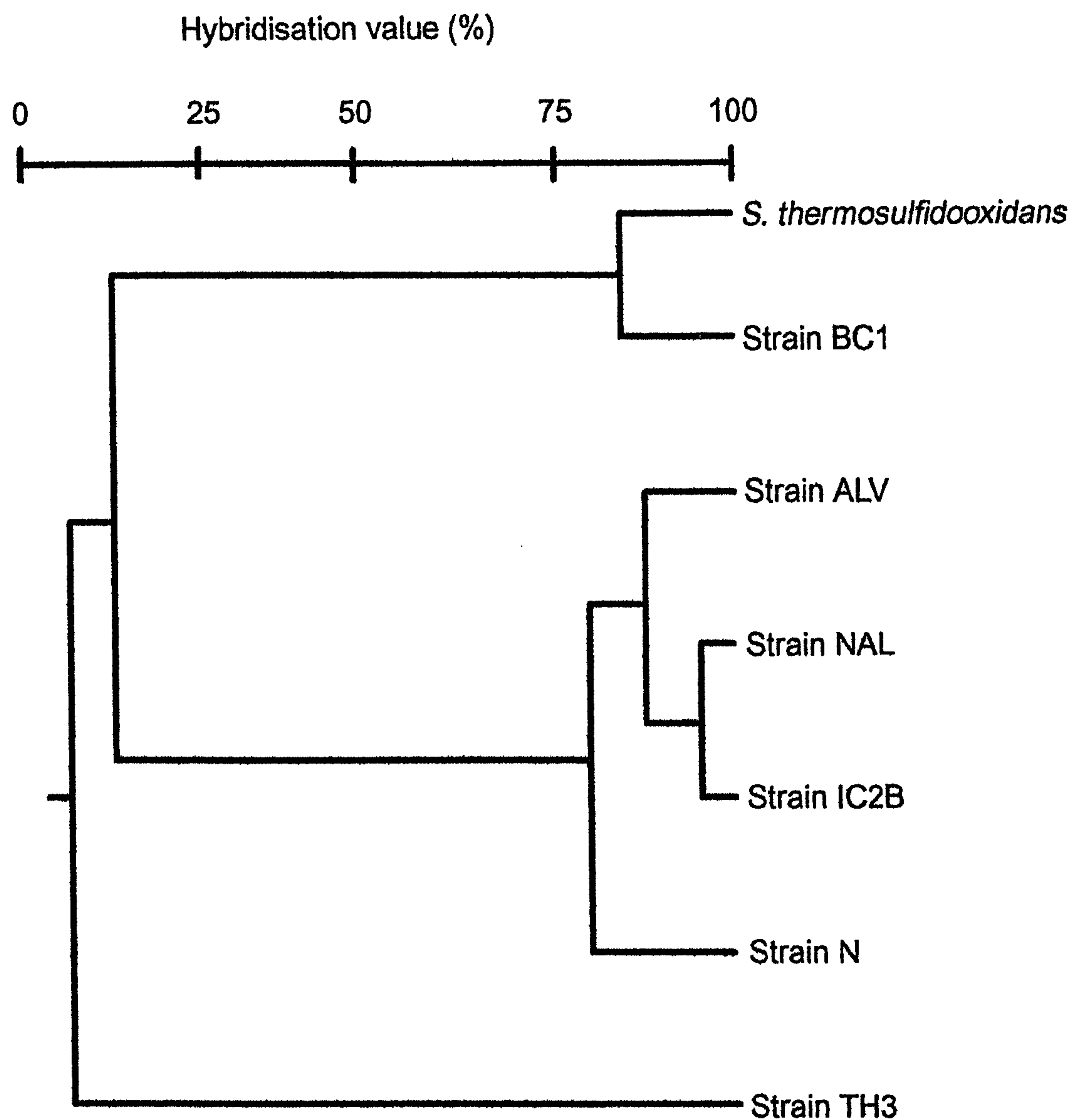


Figure 3.2: Dendrogram of moderately thermophilic iron-oxidizing bacteria based on DNA:DNA hybridisation values. The NTSYS-pc software package was used to produce the dendrogram by UPGMA clustering techniques.

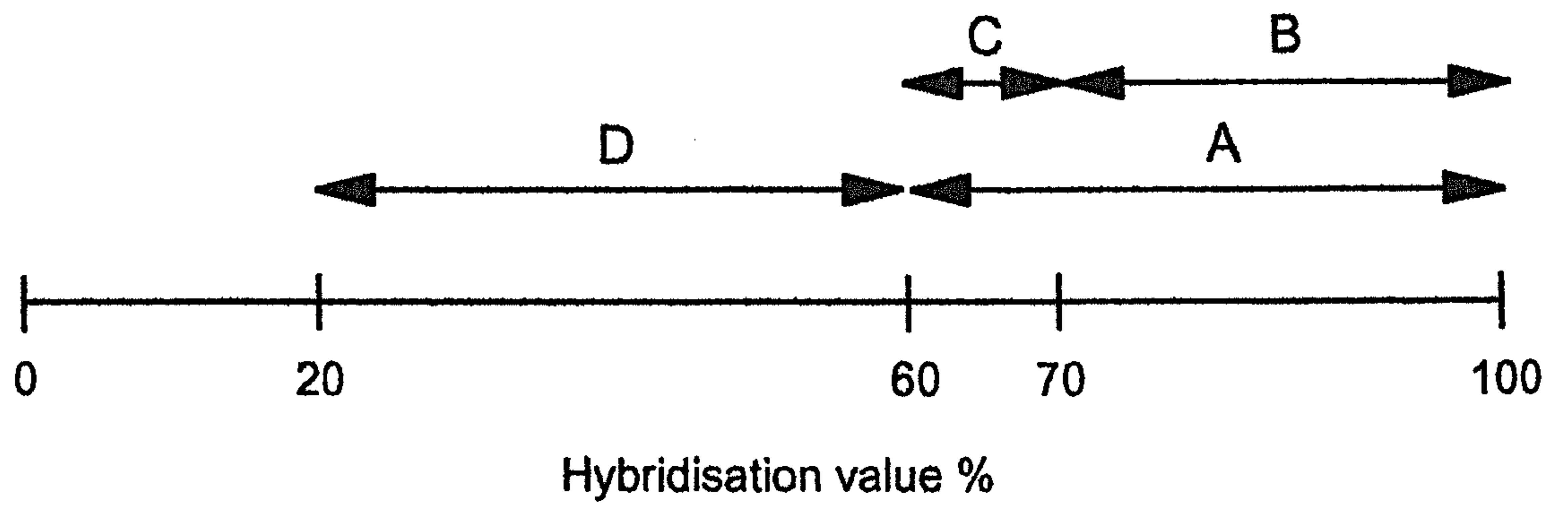
Isolate	% Hybridisation to:						
	<i>S.t.</i> ^a	Strain BC1	Strain ALV	Strain NAL	Strain IC2B	Strain N	Strain TH3
<i>S.t.</i> ^a	100	81	-	11	-	-	2
Strain BC1	90	100	8	11	7	8	4
Strain ALV	-	13	100	93	88	83	-
Strain NAL	12	10	86	100	93	79	5
Strain IC2B	-	10	82	96	100	83	-
Strain N	-	13	75	82	73	100	-
Strain TH3	3	4	-	2	-	-	100

^a *S.t.* abbreviation for *Sulfobacillus thermosulfidooxidans*.

Table 3.2: DNA:DNA hybridisation values of moderately thermophilic iron-oxidizing bacteria.

Values are the average of triplicate readings obtained from three separate experiments.

A DNA hybridisation value of 60-70% is normally considered the minimum value for strains within a species (Schleifer and Stackebrandt, 1983; Johnson, 1984). This suggests that strain BC1 and *S. thermosulfidooxidans* belong to the same species. Strains ALV, NAL, IC2B, and N also belong to the same species as each other, but not that of strain BC1 and *S. thermosulfidooxidans*. Using Johnson's scheme (Figure 3.3), these organisms can be further classified. Strain BC1 and *S. thermosulfidooxidans* are varieties within the same subspecies. Strains ALV, NAL, IC2B, and N are varieties within the same subspecies, but belong to a separate genus to strain BC1 and *S. thermosulfidooxidans*. Strain TH3 belongs to a third separate genus.



- A. 60-100% species.
- B. 70-100% varieties within a subspecies of A.
- C. 60-70% other subspecies of A.
- D. 20-60% species closely related to species A.

Figure 3.3: Relationship of organisms on the basis of DNA:DNA hybridisation values.
Reproduced from Johnson (1984).

3.3: Analysis of total cellular protein electrophoretic patterns.

As well as DNA:DNA hybridisation, a second method of comparison was used, that of whole cell protein electrophoresis fingerprinting. Providing that bacteria are grown under standardised conditions and protein samples are treated identically, then total cellular protein electrophoresis can be used for comparative purposes (Kerstens and De Ley, 1975). This technique involves the electrophoresis of proteins in the conventional way, and then the recording of the patterns obtained by densitometry or a similar method. The resulting densitometry plots are then processed to obtain groupings based on quantification of their resemblance. Computer aided pattern analysis has made this an attractive method for rapid identification of new isolates (Vauterin and Vauterin, 1992). Unfortunately computer-aided pattern analysis was not available. Therefore, a relatively simplified comparison of patterns was carried out by eye .

Conventional SDS gel electrophoresis of protein samples from a selection of isolates was carried out using 10% (w/v) acrylamide gels. The electrophoretic patterns were scanned into a computing densitometer. Densitometry traces of each sample lane were compared to each other by overlaying them on a light box. This allowed any small variations in the positions of homologous bands to be compensated for (computer analysis carries out a similar process known as normalisation). Each trace was then divided into 1 mm sections down its length, and the positions of the 20 most intense bands for that isolate were marked within these sections (a 1 was scored in a section if it contained one of the top 20 bands, and a 0 if it did not). This resulted in a similarity matrix which was analysed, using the NTSYS-pc software package, to produce a dendrogram of organism relatedness by UPGMA clustering techniques.

Figure 3.4 shows the results for the analysis of one such gel. Three gels in total were analysed by densitometry, and several more were visually inspected to ensure that a typical reproducible trace was used for each isolate. The results obtained agreed with the DNA:DNA hybridisation results, with three distinct groups. Strains ALV, NAL, IC2B, and N again grouped closely together. Strain THWX, which was not included in

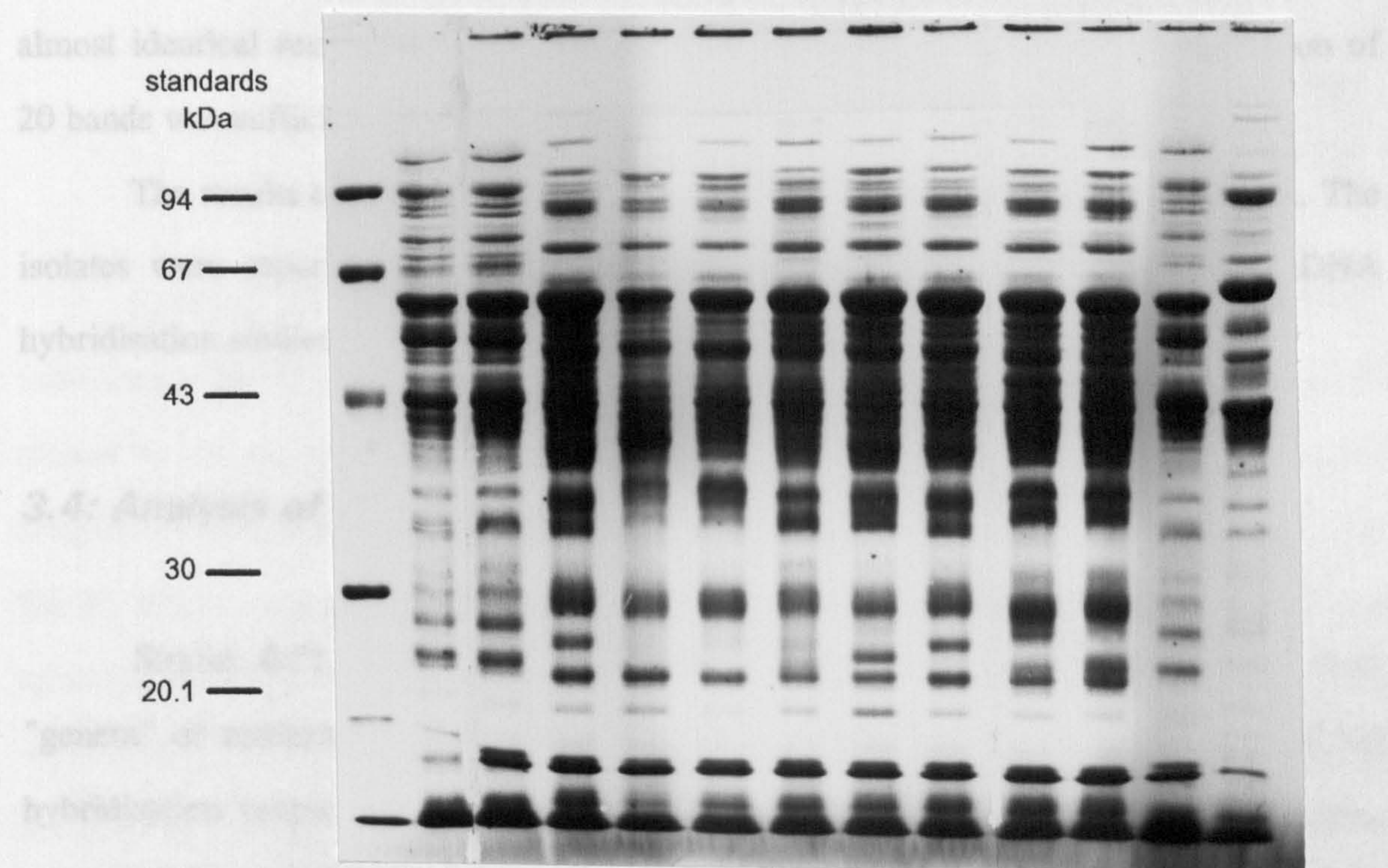
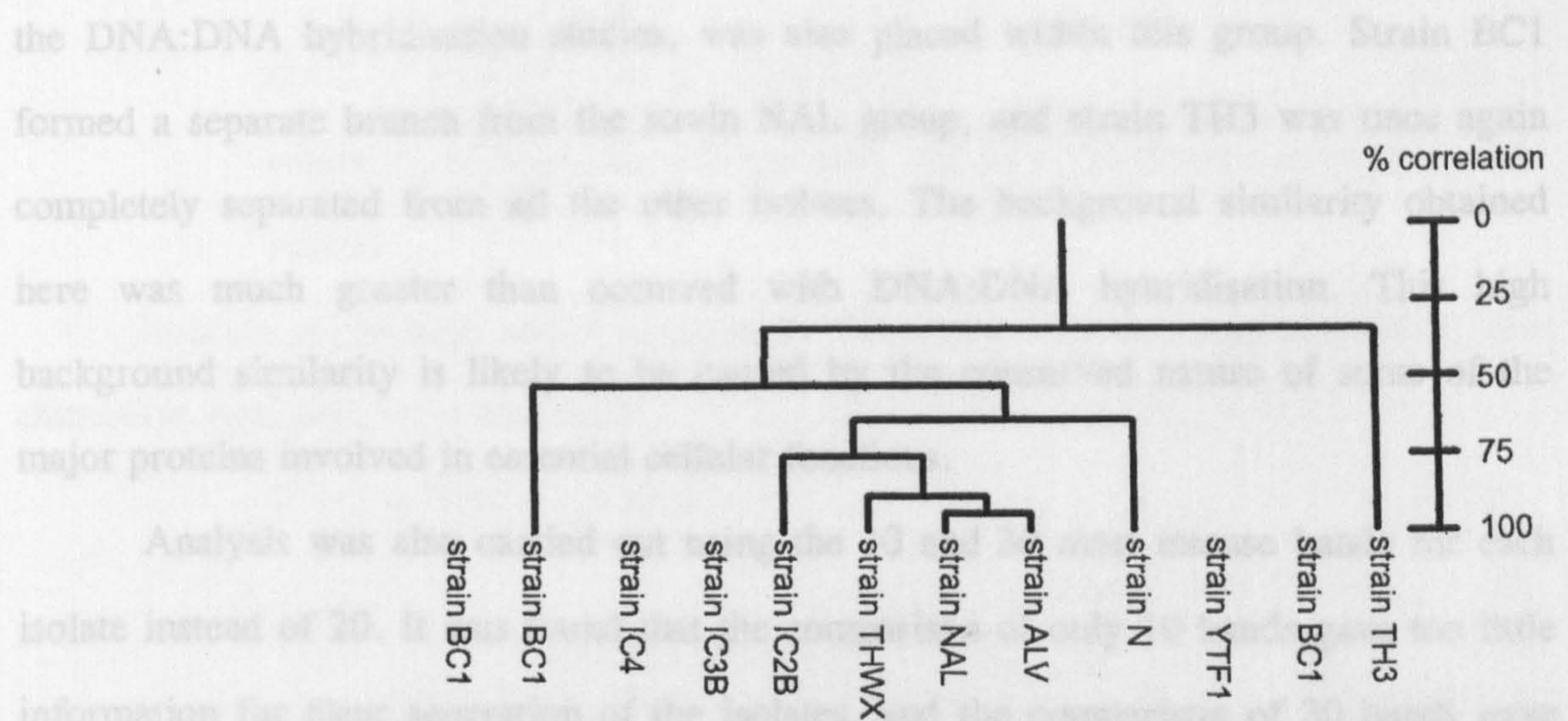


Figure 3.4: Dendrogram of moderately thermophilic iron-oxidizing bacteria based on protein profile analysis. The corresponding Coomassie stained SDS-PAGE protein profiles were analysed by densitometry. Densitometry traces of protein profiles were compared, and a similarity matrix produced. The NTSYS-pc software package was then used to produce the dendrogram by UPGMA clustering techniques. Additional gel tracks, not included in the dendrogram, are shown for comparative purposes. (Strain 3B appeared identical to strain 2B. Strain IC4 appeared almost identical to strains 2B and 3B. Strain YTF1 appeared identical to strain N).

the DNA:DNA hybridisation studies, was also placed within this group. Strain BC1 formed a separate branch from the strain NAL group, and strain TH3 was once again completely separated from all the other isolates. The background similarity obtained here was much greater than occurred with DNA:DNA hybridisation. This high background similarity is likely to be caused by the conserved nature of some of the major proteins involved in essential cellular functions.

Analysis was also carried out using the 10 and 30 most intense bands for each isolate instead of 20. It was found that the comparison of only 10 bands gave too little information for clear separation of the isolates, and the comparison of 30 bands gave almost identical results to those obtained with 20 bands, indicating that comparison of 20 bands was sufficient to enable clear separation of the different isolates.

The results confirm that this is a useful technique for comparative purposes. The isolates were separated into three main groups, as was obtained in the DNA:DNA hybridisation studies.

3.4: Analysis of DNA base composition.

Strains BC1, NAL and TH3, were selected as representatives of the three "genera" of moderately thermophilic iron-oxidizing bacteria, indicated by DNA:DNA hybridisation values. DNA was extracted from these isolates and the base composition measured by automated DNA melt testing using a Peltier temperature controller. Samples of DNA of known base composition from *Escherichia coli* strain B (G+C content 52 mol%), and *Micrococcus luteus* (G+C content 72.3 mol%) were used as standards. G+C values were calculated from the measured melting temperatures using the DNA melt software. It was found that the DNA melt software package calculated results for G+C contents of the standards about 4-5% higher than the quoted values. The equation used by this software package to calculate the G+C content was that of Mandel and Marmur (1968):

$$G+C = 2.44 (T_m - 81.5 - 16.6 \log M) \quad (3.1)$$

where T_m is the melting temperature and M is the molar concentration of cations.

Because of the high results obtained by the DNA software using equation 1, two alternative equations were also used:

$$G+C = 2.44 (T_m - 53.9) \quad (3.2)$$

(Mandel and Marmur, 1968)

$$G+C = G+C \text{ of standard DNA} + 2.08 (T_m - T_m \text{ of standard DNA}) \quad (3.3)$$

(Owen and Hill, 1979)

Equation 3.2 also gave higher values than expected for the standards. All G+C values were therefore calculated using equation 3.3, which gave values for the standards closest to the quoted values. This equation benefits from the use of reference DNA samples of known base composition in the calculations. Both the *E. coli* standard and the *M. luteus* standard were used as references enabling G+C values to be calculated twice for each isolate, and an average value obtained (Table 3.3).

The G+C values obtained for strain TH3 (68.0 mol%) and BC1 (48.2 mol%) are in close agreement with those quoted by Harrison (1986), 68.5 ± 1.4 and 50.0 ± 1.0 respectively. Isolate NAL had a G+C value of 54.9 mol% similar to that obtained for strain ALV by Harrison (56.6 ± 2.8). Evaluation of base compositions of other isolates has been carried out by J. Owen within this laboratory. The results of these evaluations indicate a clear difference in DNA base composition between the three groups. The *S. thermosulfidooxidans* group of organisms all have G+C values around 47-50 mol%. The second group (that includes strain NAL) all have a slightly higher G+C content of 54-57 mol%, and strain TH3 has a much higher G+C content of 67-68 mol%.

Sample	T _m (°C)	G + C (mol%) ^a <i>E. coli</i> standard	G + C (mol%) ^a <i>M. luteus</i> standard	Average G + C (mol%) ^b
<i>E. coli</i>	75.7	52.0	52.8	52.4
<i>M. luteus</i>	85.1	71.6	72.3	71.9
Strain BC1	73.7	47.8	48.6	48.2
Strain NAL	76.9	54.5	55.2	54.9
Strain TH3	83.2	67.6	68.4	68.0

^a G+C value calculated using equation 3.3 and the stated standard as a reference.

^b The average of the values obtained using the two reference standards is given.

Table 3.3: DNA melting temperatures and base composition of moderately thermophilic iron-oxidizing bacteria.

3.5: Morphology of moderately thermophilic iron-oxidizing bacteria.

Extensive morphological studies have been carried out on *Sulfobacillus* species. The genus *Sulfobacillus* consists of spore-forming rod-shaped bacteria similar to representatives of the genus *Bacillus* (Karavaiko *et al.*, 1988). Cell branching differs from that seen with bacilli, with pleomorphic forms similar to coryneform bacteria (Golovacheva, 1979). The named isolates vary in size between 0.5-0.9 μm in width, and 1-6 μm in length. The other moderately thermophilic iron-oxidizers have received less detailed morphological study. Isolates TH1 and TH3 are rods of sizes 0.8 μm x 1.6-3.2 μm and 0.5 μm x 1-1.6 μm respectively (Brierley, 1978).

Although this group comprises bacteria of similar morphology, all being Gram-positive rods, some differences have been noted among the different isolates. Growth conditions have a profound effect on the cell morphology of these bacteria. Both light and transmission electron microscope studies were carried out on representative isolates.

3.5.1: Phase contrast microscopy of vegetative cells.

Figure 3.5 shows the morphology of representatives of the DNA:DNA hybridisation groups during mid-exponential growth under three conditions; autotrophic growth on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate and 1% (v/v) CO₂ in air, heterotrophic growth on 0.02% (w/v) yeast extract, and chemolithoheterotrophic growth on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract. A marked difference between the three strains is clearly seen, as well as differences between the same strain grown on the different substrates.

Strain BC1 was the largest of the three isolates under all three conditions. This large cell morphology is characteristic of the *S. thermosulfidooxidans* DNA:DNA hybridisation group. Chemolithoheterotrophically growing strain BC1 ranges in size between 1-1.4 μm x 2-6 μm , with cells occurring singly or in pairs (Fig. 3.5). This group of iron-oxidizing bacteria have a much more rounded appearance when grown heterotrophically on yeast extract, strain BC1 having dimensions 1.8-2.3 μm x 1.4-1.8 μm (Fig. 3.5). Cells occur singly, in pairs or as short chains. A characteristic of all of the moderately thermophilic iron oxidizers is a tendency for smaller cell morphologies when growing autotrophically (Norris *et al.*, 1986). Autotrophically-grown strain BC1 formed small rods 0.9 μm x 2.3-2.8 μm in size, as single cells or pairs of cells (Fig. 3.5). Another feature of autotrophically-grown cells clearly visible by phase contrast was the abundance of highly refractive endospores. Spore formation occurs more readily during autotrophic growth, although sporulation does occur during heterotrophic and chemolithoheterotrophic growth also.

Strain NAL, a representative of the second DNA:DNA hybridisation group, produced smaller rods (0.9 μm x 2.8-4.0 μm) during chemolithoheterotrophic growth than strain BC1 (Fig. 3.5). This smaller morphology is again common to all members of this DNA:DNA hybridisation group (with the exception of strain ALV). None of this group produced the more rounded cells seen with strain BC1 during heterotrophic growth on yeast extract. Instead smaller rods were formed than when grown chemolithoheterotrophically, with dimensions 0.5-0.9 μm x 2.3-2.8 μm (Fig. 3.5).

Autotrophic growth on iron produced cells of similar size to those grown heterotrophically, but a large number of spore bodies were also visible (Fig. 3.5). With all three conditions strain NAL cells were slightly longer, or at least, with longer chains frequently formed. Strain TH3 has a more rapid cell division compared to the other

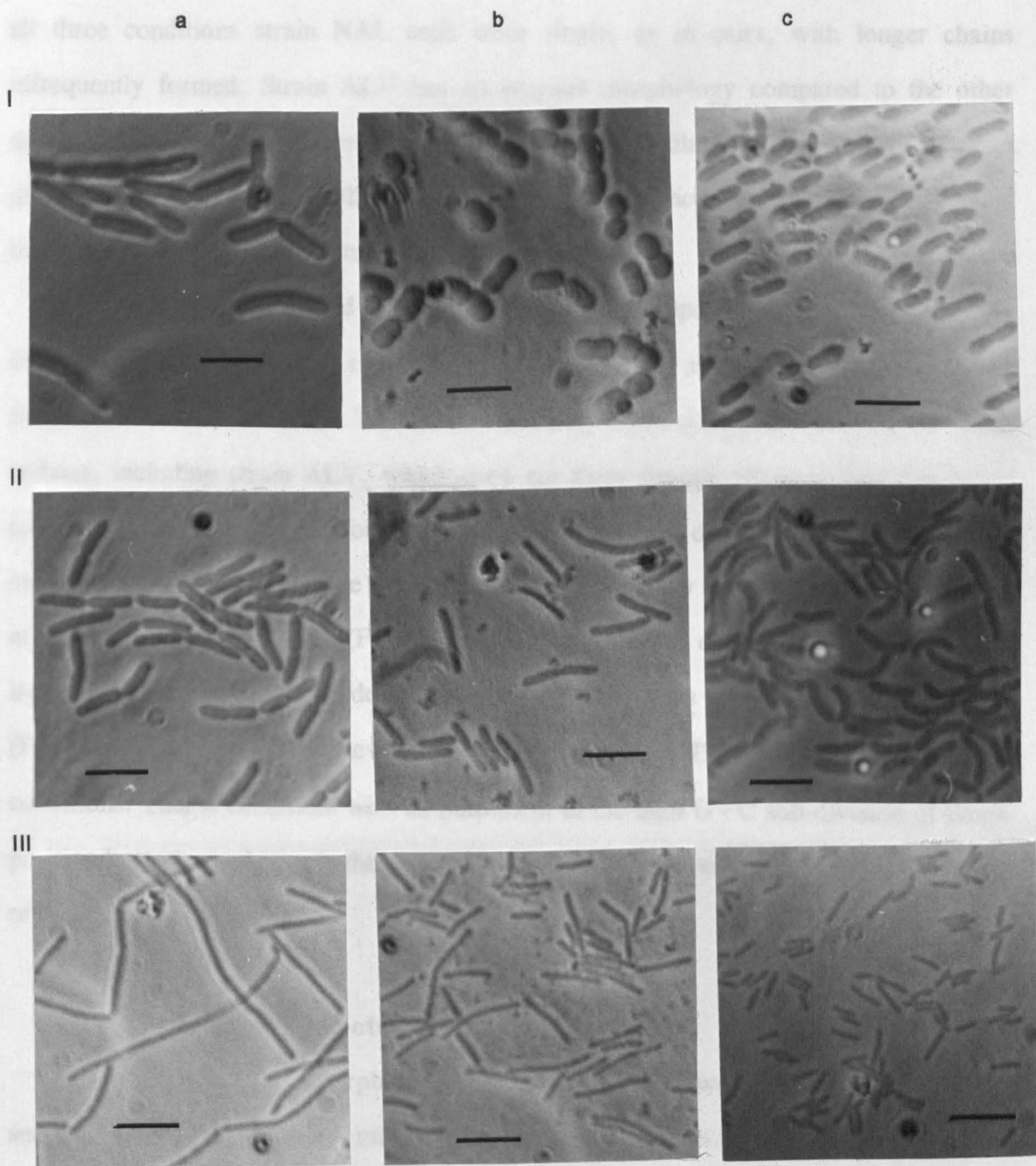


Figure 3.5: Phase contrast photomicrographs of three moderately thermophilic iron-oxidizing bacteria; strains BC1 (I), NAL (II), and TH3 (III). Bacteria were grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract (column a), heterotrophically on 0.02% (w/v) yeast extract (column b), and autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air (column c). The bar represents 5 μm.

Autotrophic growth on iron produced cells of similar size to those grown heterotrophically, but a large number of spore bodies were also visible (Fig. 3.5). With all three conditions strain NAL cells were single, or in pairs, with longer chains infrequently formed. Strain ALV has an atypical morphology compared to the other members of this hybridisation group, forming a twisted filamentous chain of cells that gives the culture an "unhealthy" appearance. This morphological difference is one of the few differences between strains ALV and NAL.

Strain TH3 had a filamentous appearance when grown chemolithoheterotrophically, consisting of cells $0.5\ \mu\text{m} \times 1-3\ \mu\text{m}$ (Fig. 3.5). This filamentous nature of strain TH3 makes it easily distinguishable from all the other isolates, including strain ALV, which does not form straight filaments and has larger cell dimensions. Strain TH3 formed filaments infrequently during heterotrophic growth on yeast extract, instead motile cells usually occurred singly or in pairs with dimensions of $0.4-0.5\ \mu\text{m} \times 1.5-3.0\ \mu\text{m}$ (Fig. 3.5). Very poor growth occurred autotrophically on iron resulting in a low cell density of small, single cells $0.3-0.4\ \mu\text{m} \times 1.0-2.0\ \mu\text{m}$ (Fig 3.5). Endospores were never observed with strain TH3 under any of the growth conditions. This is consistent with its placement in the high G+C sub-division of Gram-positive bacteria, and is a further indication of the distinctiveness of this strain from all of the other isolates.

3.5.2: Transmission electron microscopy.

Differences in cell morphology between the isolates were also evident from thin section transmission electron micrographs. All strains were grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate and 1% (v/v) CO_2 in air, except strain TH3 which was grown chemolithoheterotrophically on 50 mM ferrous iron plus 0.02% (w/v) yeast extract. Strain BC1 had a very granular appearance, with electron dense material at the poles of the cell (Figure 3.6). Polyphosphate granules have been reported at the poles of *S. thermosulfidooxidans* cells by Golovacheva (1979). However, no spherical granules of the size reported by Golovacheva (240-260 nm) were

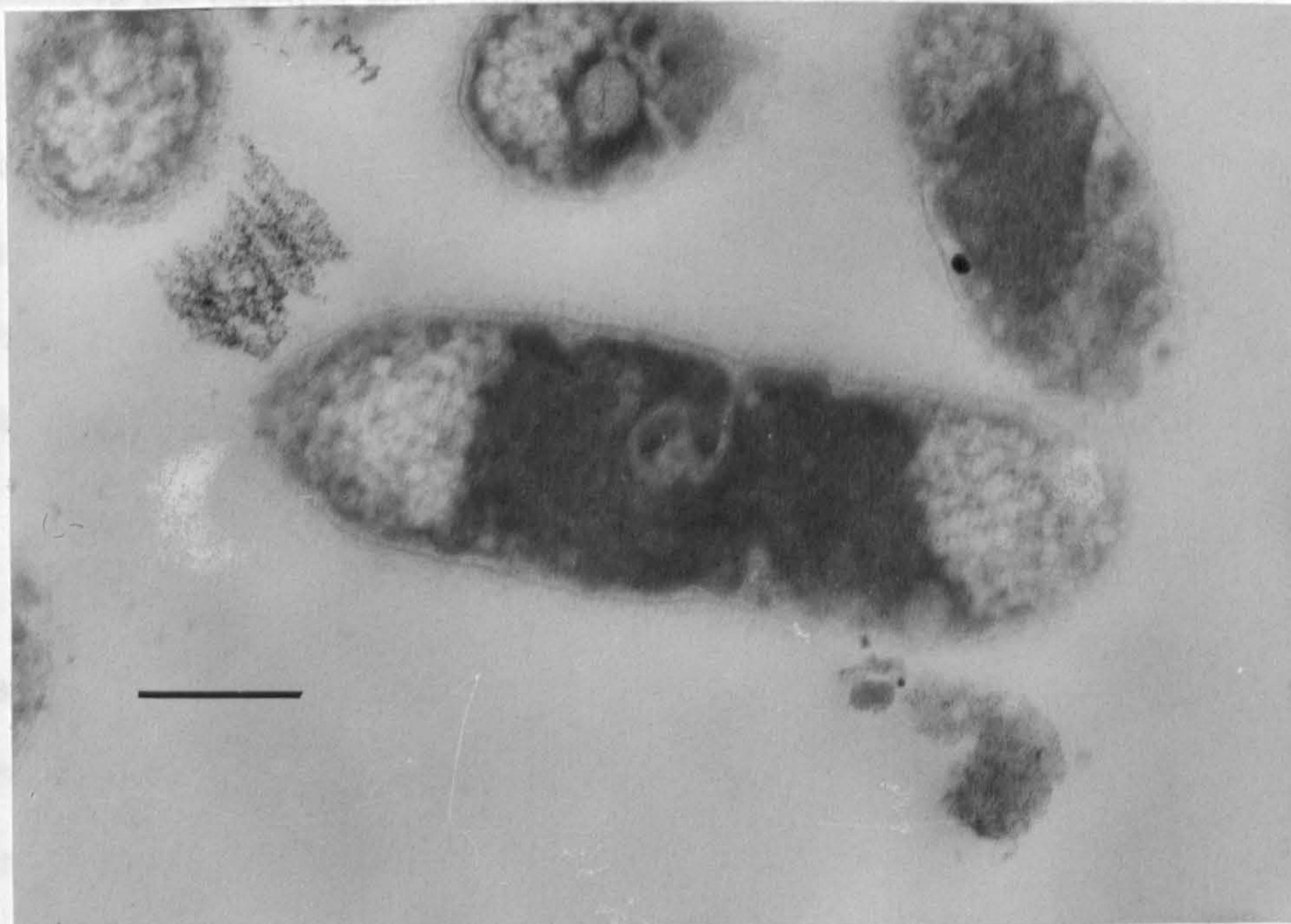


Figure 3.6: Thin section electron micrograph of strain BC1. Strain BC1 was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air. Thin sections were stained with uranyl acetate prior to transmission electron microscope analysis. Bar represents 0.5 μ m.

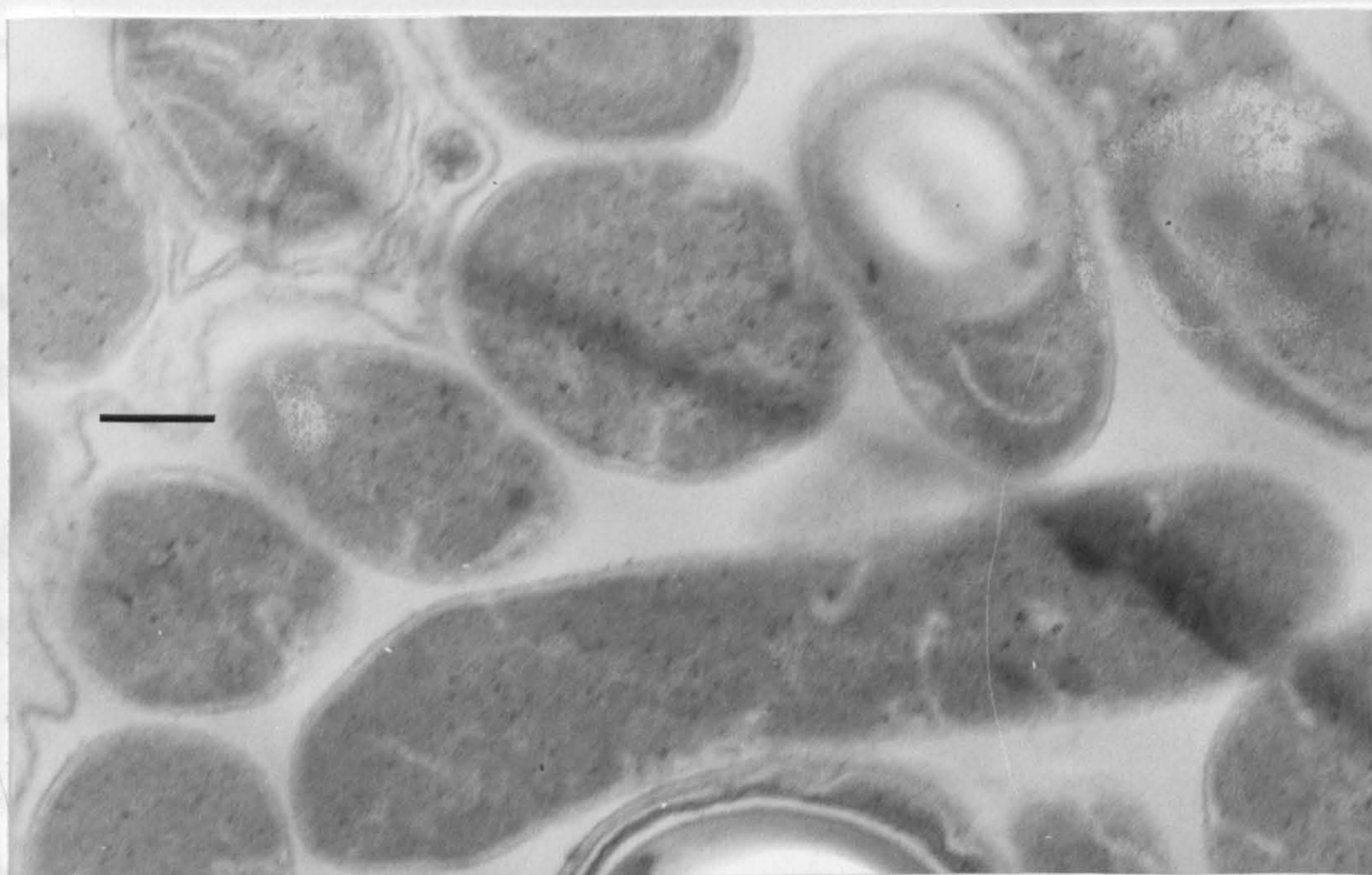


Figure 3.7: Thin section electron micrograph of strain NAL. Growth conditions and thin section analysis as described for Fig. 3.6. Bar represents 0.5 μ m.

visible in strain BC1. During the studies of *S. thermosulfidooxidans* carried out by Golovacheva the cultures were supplemented with yeast extract, under which conditions strain BC1 often contained spherical granules 240-260 nm in diameter. It is known that all prokaryotic organisms produce polyphosphate granules during exponential growth if the medium contains sufficient phosphate (Shively, 1974). This suggests that autotrophic conditions do not allow sufficient phosphate levels for polyphosphate granule formation by strain BC1, whereas chemolithoheterotrophic conditions do.

Autotrophically-grown strain NAL produced thinner rods than strain BC1 (as noted from phase contrast microscopy), and cells did not have the granular appearance of strain BC1 (Figure 3.7). Strain ALV had a similar cellular appearance to strain NAL (Figure 3.8). Strain ALV could be clearly distinguished as the cells were slightly bent rods in long twisted chains. Thin sectioning enabled only a small fraction of the chains to be seen at any one time.

As previously noted strain TH3 produced filaments of cells when grown chemolithoheterotrophically. These filaments are easily differentiated from the bent and twisted chains of strain ALV, because strain TH3 cells are much straighter, and thinner (Figure 3.9). Electron dense granules are typically seen at one pole of strain TH3 cells. These granules are about 200 nm in diameter and could be polyphosphate granules, as observed with *S. thermosulfidooxidans* (Golovacheva, 1979). Confirmation of polyphosphate granules in strain TH3 has not been established, but the localisation of these granules in the polar regions appears to be a typical phenomenon, and is consistent with the polyphosphate granules of *S. thermosulfidooxidans*.

Endospore formation has been noted with all the strains except TH3. The spores produced by all the other strains are similar. They are produced terminally or subterminally with distension of the sporangia. Distension tends to be slight in members of the *S. thermosulfidooxidans* hybridisation group (Figure 3.10), but is much greater with the strain NAL hybridisation group (Figure 3.11). Spores are spherical or slightly oval with dimensions ranging from 1.0-3.0 μm x 1.0-3.0 μm . The spore structure and



Figure 3.8: Thin section electron micrograph of strain ALV.

Growth conditions and thin section analysis as described for Fig. 3.6. Bar represents 0.5 μm .



Figure 3.9: Thin section electron micrograph of strain TH3.

Strain TH3 was grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract. Thin section analysis as for Fig. 3.6. Bar represents 0.5 μm .



Figure 3.10: Thin section electron micrograph of strain BC1 showing slight distension of sporangia, during spore formation. Growth conditions and thin section analysis as for Fig. 3.6. Bar represents 0.25 μm .



Figure 3.11: Thin section electron micrograph of strain NAL, showing large distension of sporangia during spore formation. Growth conditions and thin section analysis as for Fig. 3.6. Bar represents 0.5 μm .

formation is typical for endospore-forming bacteria such as *Bacillus spp.*. In the early stages of spore development the forespore becomes cut off from the vegetative cell, and slight cell distension occurs (Figure 3.12). In the terminal stages of spore formation, the mature spore becomes surrounded by a cortex and spore coat, but is still enclosed by the mother cell (Figure 3.13). The spore structure is seen more clearly in Figure 3.14, with the spore periplasm, germ wall, cortex, and spore coat all visible.

Despite the apparent similarity of many of the moderately thermophilic iron-oxidizers, clear differences in morphology can be demonstrated. The *S. thermosulfidooxidans* group of isolates have the largest cells dimensions. Strain BC1 (and at least one other isolate, strain ICH) have a tendency to form short fat rods which become almost coccoid during heterotrophic growth, and they display only slight sporangia distension during spore formation. The NAL group of isolates form thinner rods than the *S. thermosulfidooxidans* group, cell shape does not alter significantly during heterotrophic growth, and sporangia distension is normally large during spore formation. Strain TH3 is distinct morphologically from all the other isolates. Its cells are smaller than any other isolates and they usually form straight filaments. Spore formation has never been seen in strain TH3.

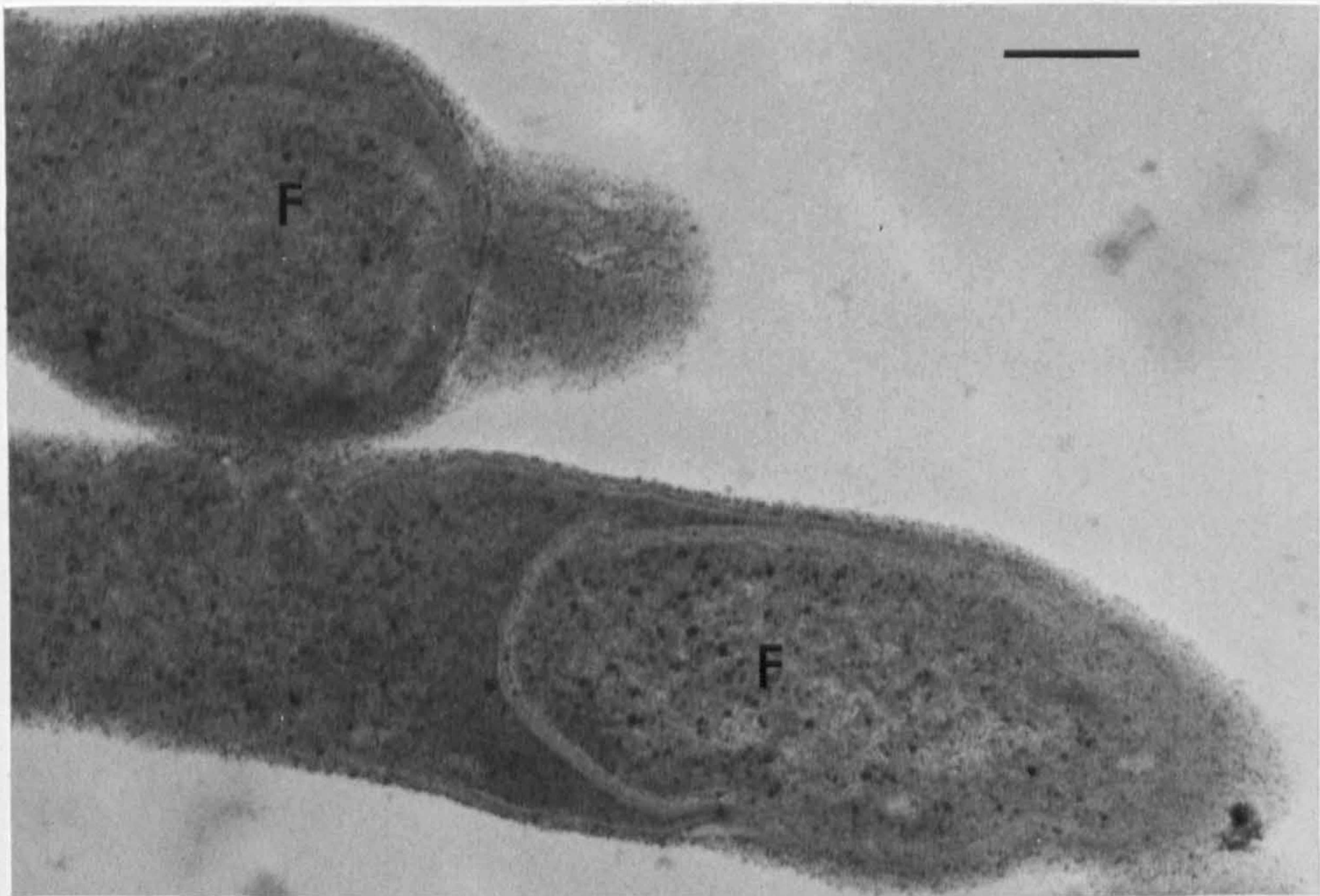


Figure 3.12: Thin section electron micrograph of strain NAL in early stages of endospore formation, with forespore visible (F). Growth conditions and thin section analysis as for Fig. 3.6. Bar represents 0.2 μm .

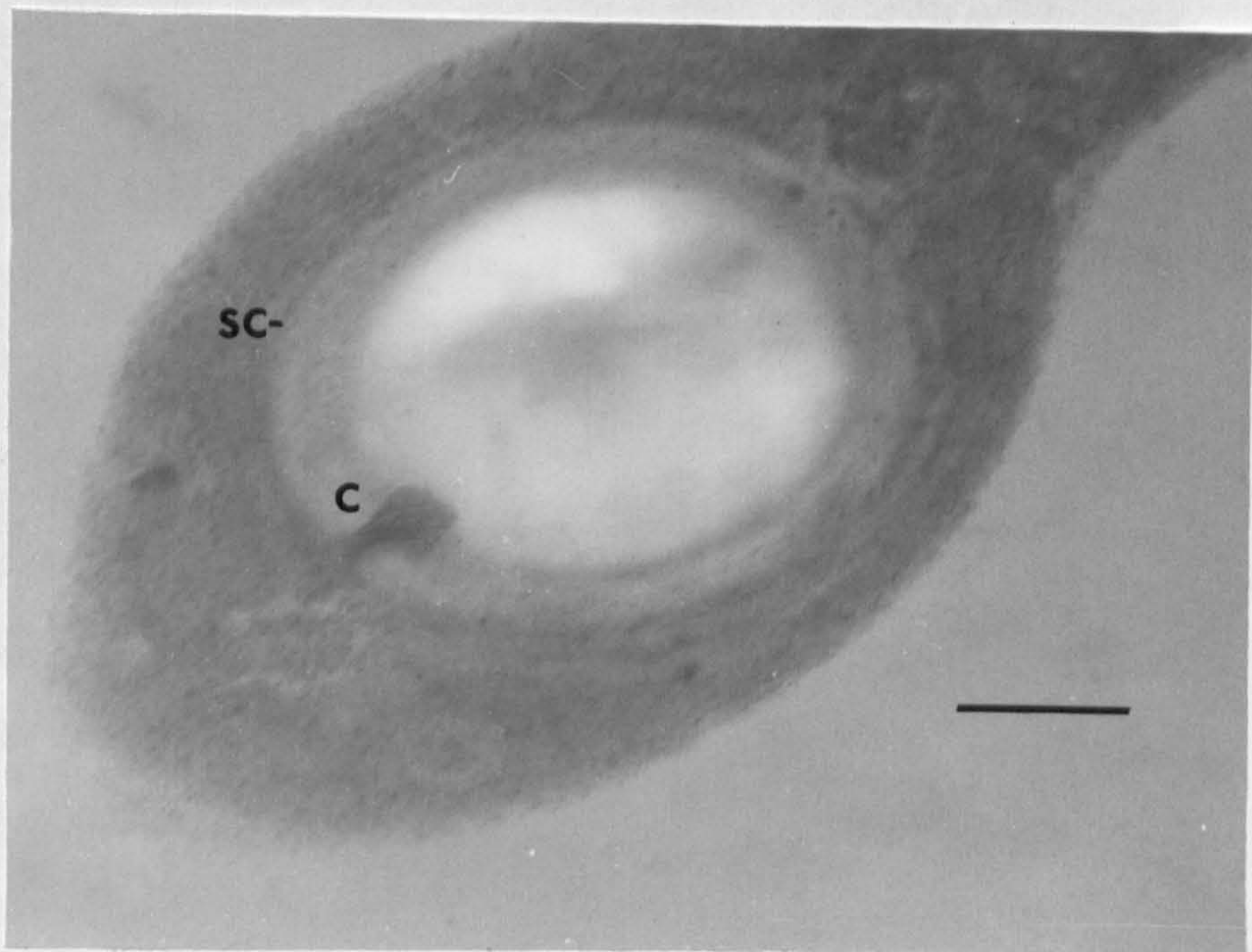


Figure 3.13: Thin section electron micrograph of strain THWX in late stages of endospore formation. The mature spore is surrounded by the spore coat (sc), and cortex (c). Growth conditions and thin section analysis as for Fig. 3.6. Bar represents 0.2 μm .

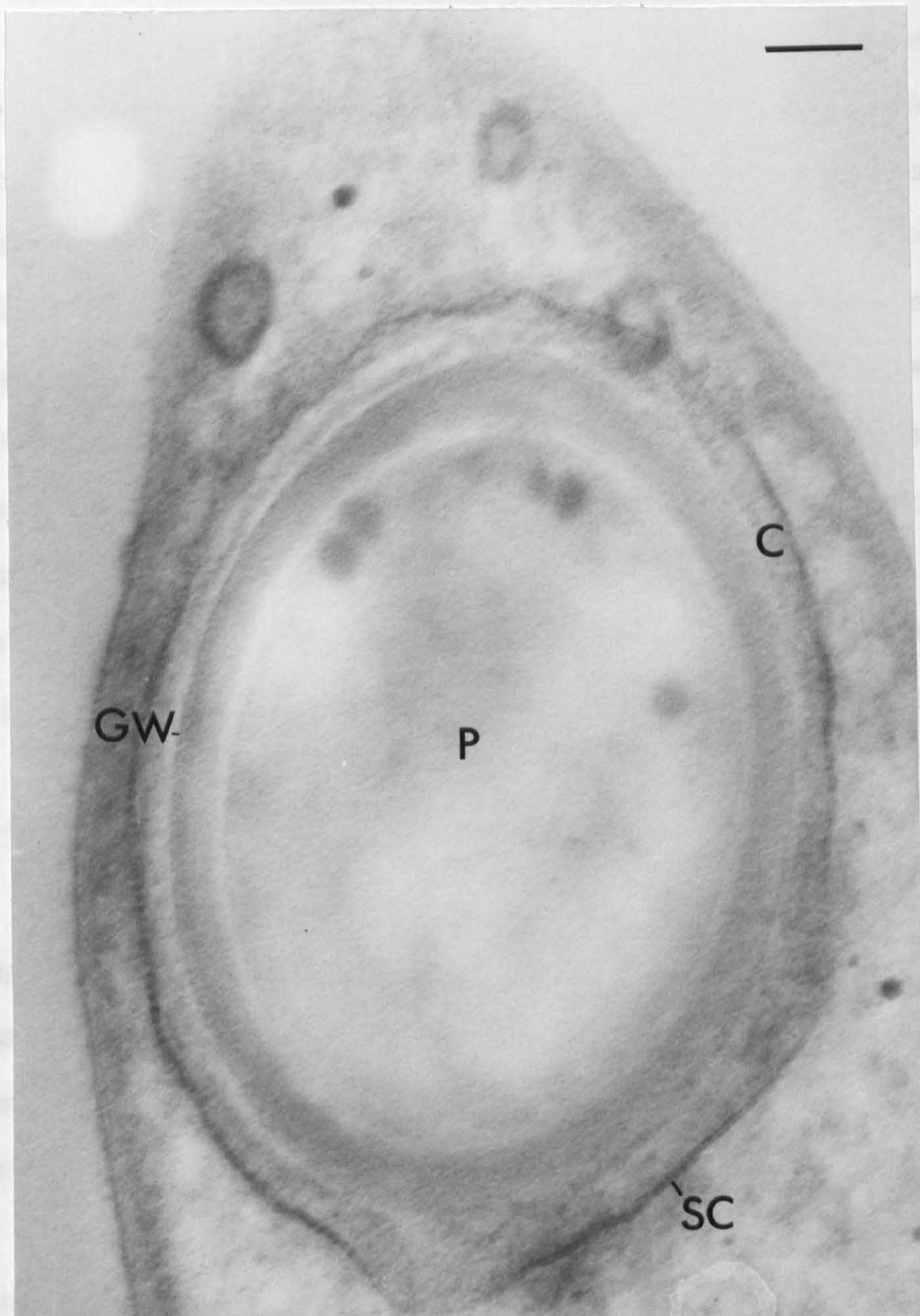


Figure 3.14: Thin section electron micrograph showing mature endospore. The spore protoplast (P), germ wall (GW), cortex (C), and spore coat (SC) are all visible. Growth conditions and thin section analysis as for Fig. 3.6. Bar represents 0.1 μm .

3.6: Physiology of moderately thermophilic iron-oxidizing bacteria.

The moderately thermophilic iron-oxidizing bacteria have optimum growth temperatures between 45-50 °C (Norris, 1989). All are probably capable of autotrophic, mixotrophic and heterotrophic growth. They grow less readily on sulphur than the acidophilic thiobacilli (Norris *et al.*, 1986), but some show better growth than others. Strain TH1, a *S. thermosulfidooxidans* group isolate, grows poorly on sulphur, even when supplemented with yeast extract, whereas strain ALV, a member of the second DNA:DNA hybridisation group grows relatively well autotrophically on sulphur (Norris *et al.*, 1986). It has been found that all the *S. thermosulfidooxidans* DNA:DNA hybridisation group, along with strain TH3, grow poorly on sulphur, whilst the NAL hybridisation group all grow well. Further differences are also seen when the isolates are grown autotrophically on iron and heterotrophically on yeast extract.

3.6.1: Autotrophic growth on ferrous iron.

During chemolithoheterotrophic growth on 50 mM ferrous iron, supplemented with yeast extract, little difference was seen between the rates of iron oxidation produced by the different isolates. However during autotrophic growth on iron, at enhanced CO₂ levels, differences between the three DNA:DNA hybridisation groups were more evident (Figure 3.15). This graph shows that the two isolates belonging to the *S. thermosulfidooxidans* hybridisation group (*S. thermosulfidooxidans* and LM1) maintained higher rates of iron oxidation. The NAL DNA:DNA hybridisation group organisms, strains NAL and ALV, had a slightly slower rate of iron oxidation. This difference in maximal iron oxidation rates is typical for all members of the two DNA:DNA hybridisation groups. Strain TH3 grew very poorly autotrophically, as had been noted previously (Norris and Barr, 1985). Only about 20% of the ferrous iron was oxidized by strain TH3, but the organism could be maintained by serial sub-culturing under these conditions.

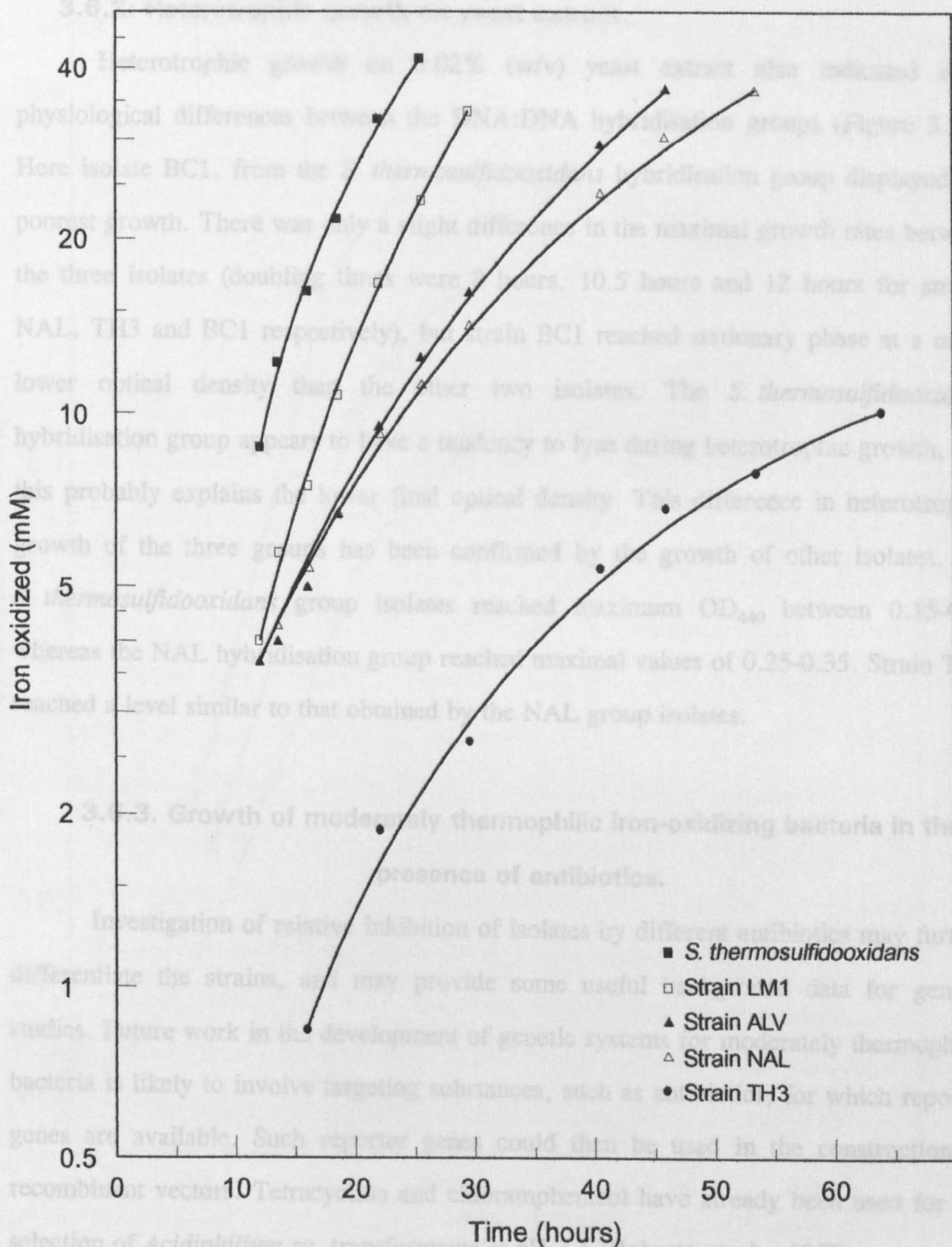


Figure 3.15: Iron oxidation during autotrophic growth of five moderately thermophilic iron-oxidizing bacteria in pH 1.7 medium containing 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air at 48 °C.

3.6.2: Heterotrophic growth on yeast extract.

Heterotrophic growth on 0.02% (w/v) yeast extract also indicated some physiological differences between the DNA:DNA hybridisation groups (Figure 3.16). Here isolate BC1, from the *S. thermosulfidooxidans* hybridisation group displayed the poorest growth. There was only a slight difference in the maximal growth rates between the three isolates (doubling times were 8 hours, 10.5 hours and 12 hours for strains NAL, TH3 and BC1 respectively), but strain BC1 reached stationary phase at a much lower optical density than the other two isolates. The *S. thermosulfidooxidans* hybridisation group appears to have a tendency to lyse during heterotrophic growth, and this probably explains the lower final optical density. This difference in heterotrophic growth of the three groups has been confirmed by the growth of other isolates. All *S. thermosulfidooxidans* group isolates reached maximum OD₄₄₀ between 0.15-0.2, whereas the NAL hybridisation group reached maximal values of 0.25-0.35. Strain TH3 reached a level similar to that obtained by the NAL group isolates.

3.6.3. Growth of moderately thermophilic iron-oxidizing bacteria in the presence of antibiotics.

Investigation of relative inhibition of isolates by different antibiotics may further differentiate the strains, and may provide some useful background data for genetic studies. Future work in the development of genetic systems for moderately thermophilic bacteria is likely to involve targeting substances, such as antibiotics, for which reporter genes are available. Such reporter genes could then be used in the construction of recombinant vectors. Tetracycline and chloramphenicol have already been used for the selection of *Acidiphilium* sp. transformants at pH 3.5 (Roberto *et al.*, 1989).

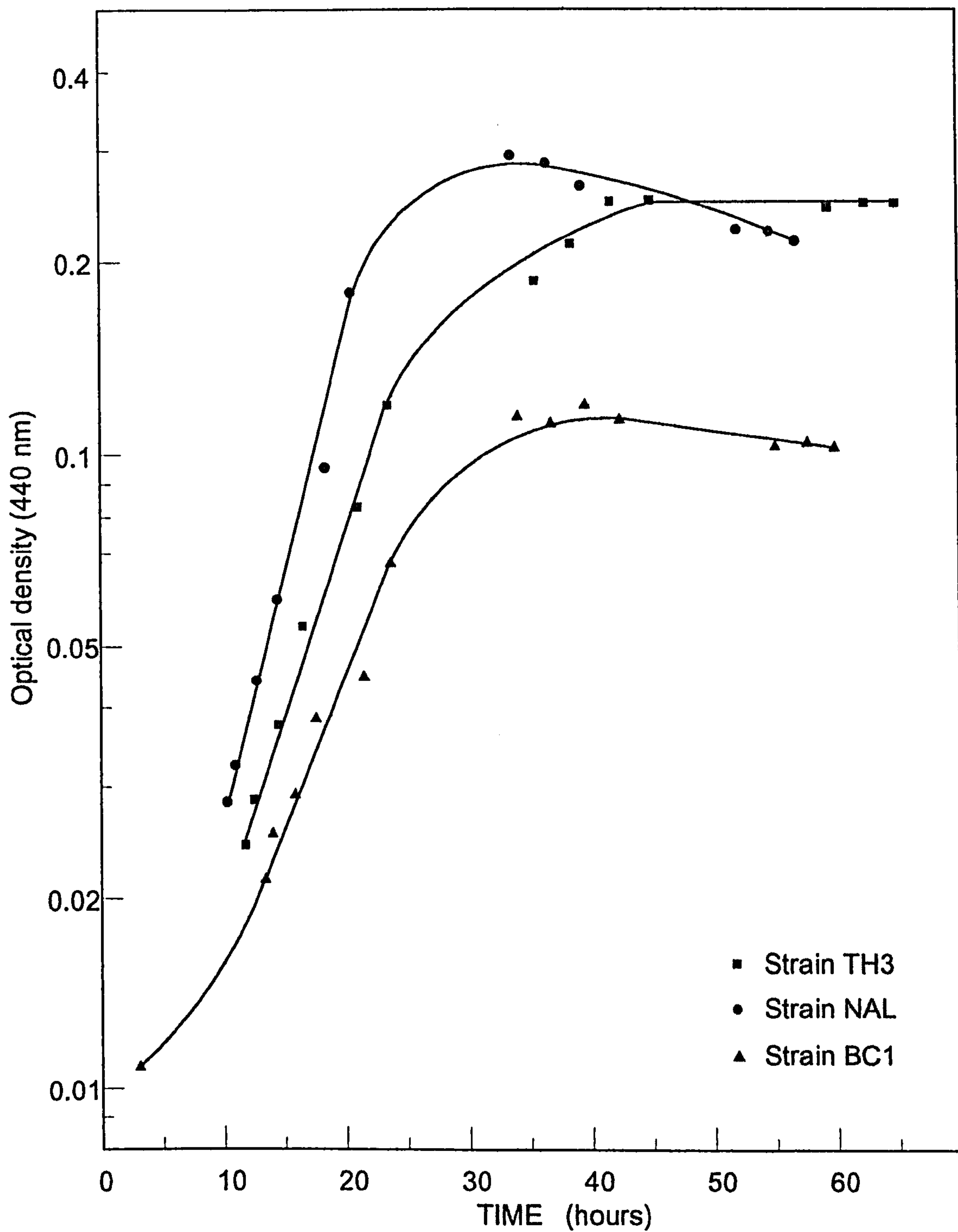


Figure 3.16: Heterotrophic growth of three moderately thermophilic iron-oxidizing bacteria in pH 2.0 medium containing 0.02% (w/v) yeast extract, at 48 °C.

The most convenient method for testing a number of bacteria against a range of antibiotics is using disc diffusion methods. A disc containing a known amount of antibiotic is placed on the assay plates seeded with the test organism. The plates are then incubated, and zones of inhibition measured around the disc. Since antibiotics differ in their diffusion rates through solid media, the inhibition zone size cannot be used directly to compare potency of different antibiotics to one another, and it cannot be assumed that one organism is more susceptible to one antibiotic than another solely on the basis of zone size. The low pH and high temperatures are also likely to affect antibiotics to different extents, making direct comparisons between antibiotics difficult. However, the inhibition produced by each antibiotic with a range of organisms grown and tested under identical conditions can be compared.

Pour plates of each organism were prepared using 1% (v/v) inoculum of exponentially growing cells. All moderately thermophilic cultures were grown chemolithoheterotrophically on ferrous iron plus yeast extract, whilst the mesophile *Thiobacillus ferrooxidans* was grown autotrophically on ferrous iron. The solid medium consisted of salts at pH 2.2 and 0.5% (w/v) agarose type II, supplemented with 20 mM ferrous iron and 0.02% (w/v) yeast extract (except for *T. ferrooxidans* plates which contained no yeast extract). 20 ml medium was used per plate, and all plates were incubated inverted at their respective growth temperatures after assay discs had been placed on the plates. *T. ferrooxidans* plates were incubated at 30 °C, whilst the moderately thermophilic cultures were incubated at 48 °C. Plates were checked daily and zones of inhibition measured as soon as they were visible. Two zones were measured wherever possible; a zone of complete growth inhibition, and a zone of iron oxidation inhibition. However, some of the isolates grew so poorly on solid media that only the zone of iron oxidation inhibition could be measured. Iron oxidation inhibition was clearly visible, as oxidation results in an orange/brown ferric hydroxide precipitation within the agar. The radius of a zone was measured from the edge of the assay disc to the periphery of a zone.

The antibiotics used in the assays are shown in table 3.4. An indication of each antibiotic's site of action within the cell is given, along with the type of antibiotic and its general range of activity. Certain types of antibiotics tend to be more susceptible to heat and acid inactivation than others. Aminoglycosides, chloramphenicol, and tetracyclines are all relatively heat and acid stable. Macrolides are heat stable, but sensitive to acid. Penicillins and fusidic acid tend to be sensitive to both heat and acid, however, the different penicillin sidechains alter stabilities (e.g. methicillin is acid stable due to its sidechain).

After 7 days incubation all plates had been measured. Figures 3.17 a.-m. show the inhibition zones obtained for each antibiotic with the isolates tested.

25 μ g chloramphenicol produced large zones of iron oxidation inhibition and growth inhibition (where visible) with all the moderately thermophilic iron-oxidizers, in comparison to the mesophile *T. ferrooxidans* (Fig. 3.17 a.). Little difference was seen in resistance to chloramphenicol between the three DNA:DNA hybridisation groups of moderately thermophilic organisms.

25 μ g colistin sulphate produced a small inhibitory effect (Fig. 3.17 b.). Only iron oxidation inhibition zones were visible. Strain TH3 was the most sensitive isolate tested, and with the exception of strain ALV the second DNA:DNA hybridisation group appeared less sensitive than the *S. thermosulfidooxidans* hybridisation group. It should also be noted that several of the Gram-positive moderate thermophiles were more sensitive than the Gram-negative mesophile *T. ferrooxidans*, despite this antibiotic's main activity being against Gram-negative bacteria.

Erythromycin produced relatively large zones of inhibition for all of the isolates, including *T. ferrooxidans* (Fig. 3.17 c.). However there was no clear relationship between inhibition and DNA:DNA hybridisation group.

Fusidic acid produced even larger zones than erythromycin for all the isolates, but once again no clear differences between inhibition of the three DNA:DNA hybridisation groups were seen (Fig. 3.17 d.).

Antibiotic	Classification	Activity Range	Site of Action	Amount Used (μg)
Chloramphenicol	Benzene derivative	G + /G-	Protein synthesis	25
Colistin sulphate	Polymyxin	G-	Cell membrane	25
Erythromycin	Macrolide	<u>G +</u> /G-	Protein synthesis	5
Fusidic acid	Steroid	G +	Protein synthesis	10
Novobiocin	Aromatic ether	<u>G +</u> /G-	DNA replication	5
Tetracycline	Tetracycline	G + /G-	Protein synthesis	25
Thiostrepton	Peptide	G +	Protein synthesis	20
Methicillin	Penicillin	<u>G +</u> /G-	Peptidoglycan synthesis	10
Mezlocillin				30
Ticarcillin				75
Amikacin	Aminoglycoside	G + /G-	Protein synthesis	30
Gentamycin				10
Streptomycin				10

Table 3.4: Characteristics of antibiotics used in disc assays.

The spectrum of bacterial activity is indicated by G + for Gram-positive, and G- for Gram-negative, where one of these is underlined the antibiotic is more active against that bacterial type. The sites of action are given either within the bacterial cell, or the metabolic pathway affected. The amount of antibiotic used per assay disc is indicated in μg .

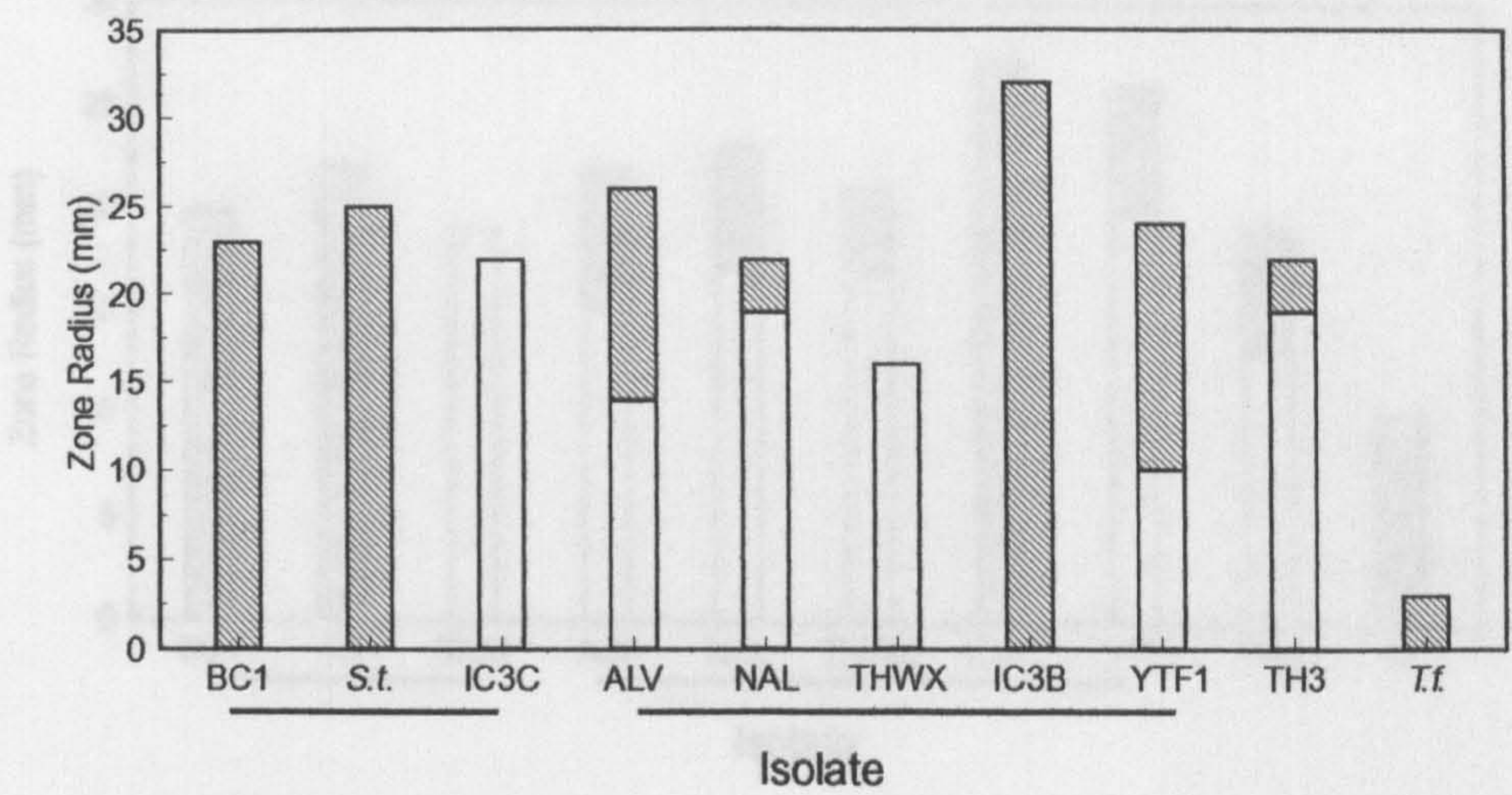
Figure 3.17: Zones of growth and iron oxidation inhibition for moderately thermophilic iron-oxidizing isolates in antibiotic disc diffusion assays.

Five pages of graphs illustrate the sizes of zones of growth inhibition and iron oxidation inhibition (mm) by antibiotic discs (Mast Laboratories, Liverpool). The radius of the growth inhibition zone was measured from the edge of the disc to the periphery of the zone (empty bar). Iron oxidation inhibition zone was measured from the edge of the growth inhibition zone to the periphery of the iron oxidation zone (shaded bar). Growth zones were often hard to identify, due to the relatively poor growth of these organism on solid media. Iron oxidation was clearly visible by the production of ferric hydroxide precipitates within the medium. The lack of a growth inhibition zone may, therefore, indicate that the zone was not clearly visible rather than was completely absent. Alternatively it may indicate that iron oxidation and growth were inhibited to the same extent.

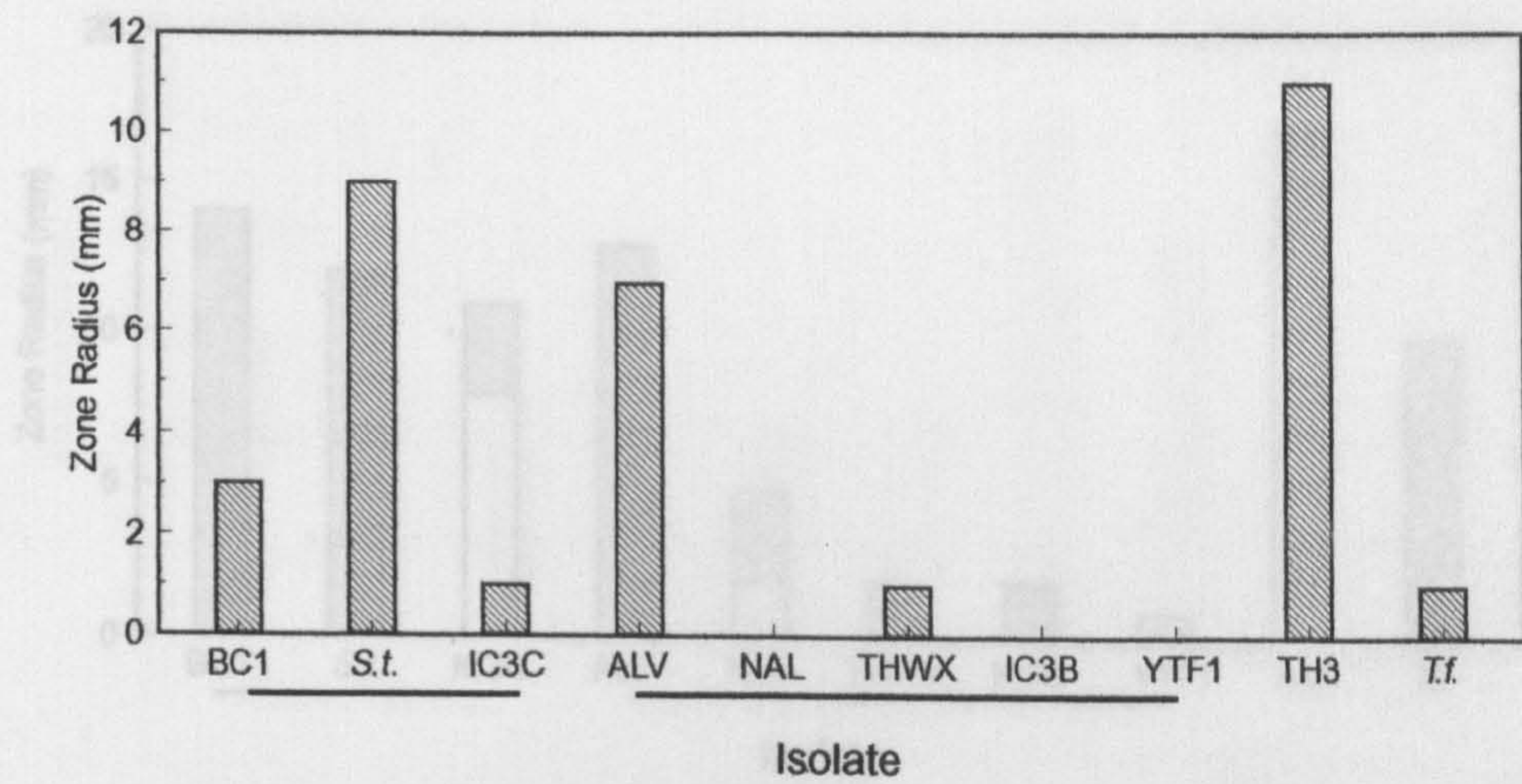
All zones were measured after 7 days incubation. All moderate thermophile plates were incubated at 48 °C, whilst *Thiobacillus ferrooxidans* plates were incubated at 30 °C. The isolates tested were as follows:

BC1	Moderate thermophile strain BC1.
<i>S.t.</i>	Moderate thermophile <i>Sulfobacillus thermosulfidooxidans</i> .
IC3C	Moderate thermophile strain IC3C.
ALV	Moderate thermophile strain ALV.
NAL	Moderate thermophile strain NAL.
THWX	Moderate thermophile strain THWX.
IC3B	Moderate thermophile strain IC3B.
YTF	Moderate thermophile strain YTF.
TH3	Moderate thermophile strain TH3.
<i>T.f.</i>	Mesophile <i>Thiobacillus ferrooxidans</i> .

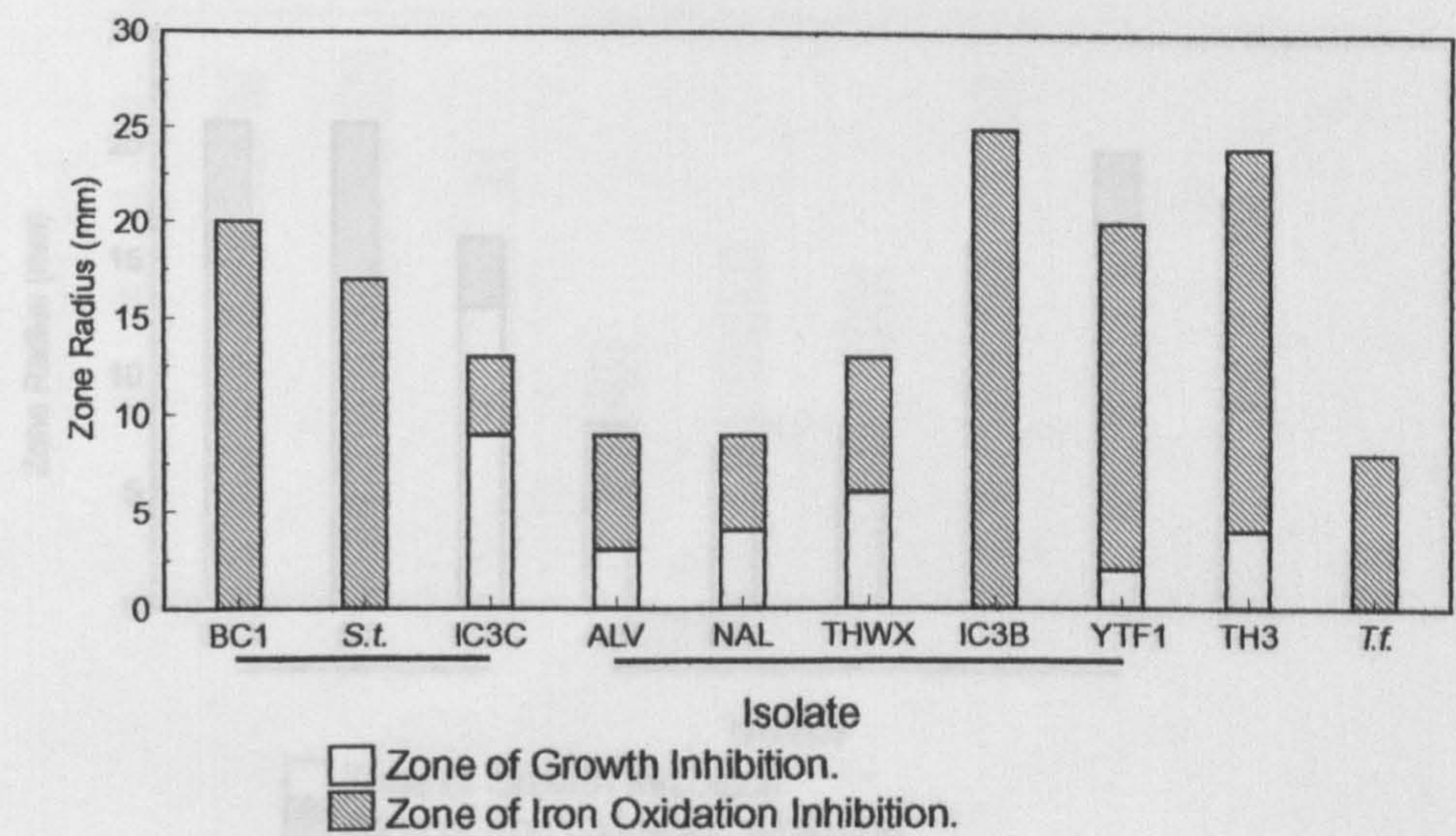
3.17 a: Chloramphenicol (25 µg)



3.17 b: Colistin Sulphate (25 µg)

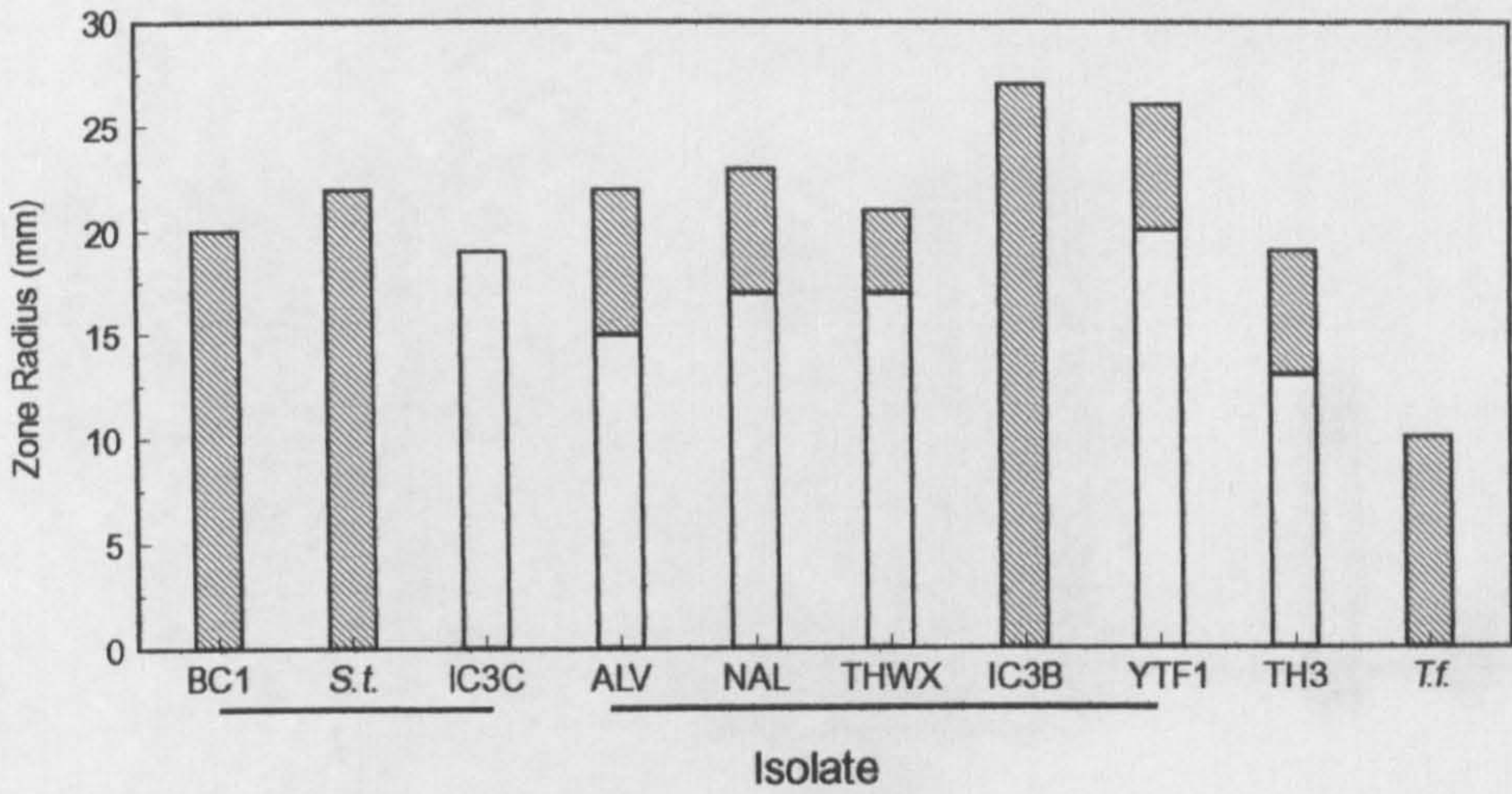


3.17 c: Erythromycin (5 µg)

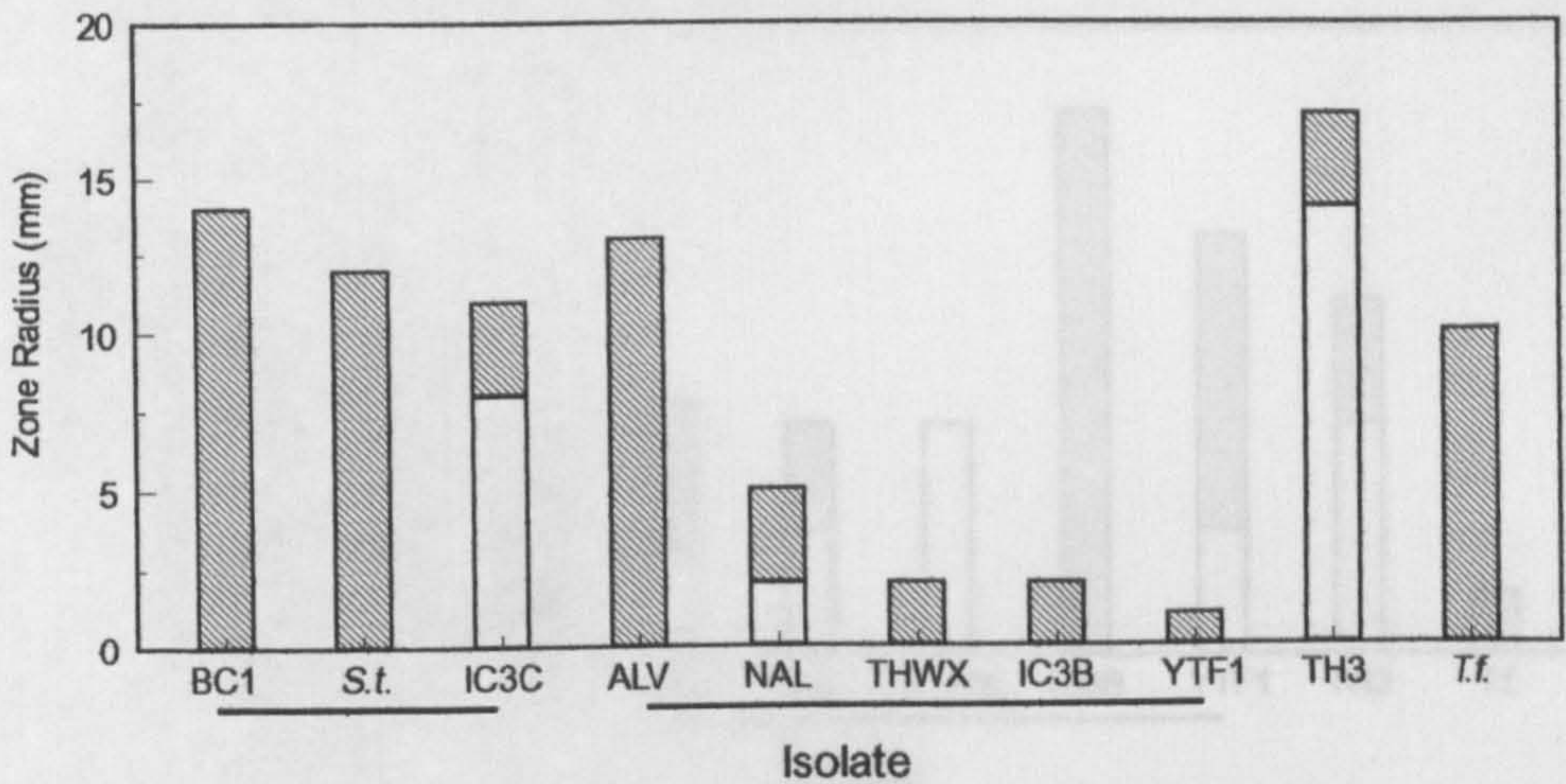


— Isolates from the same DNA:DNA hybridisation group.

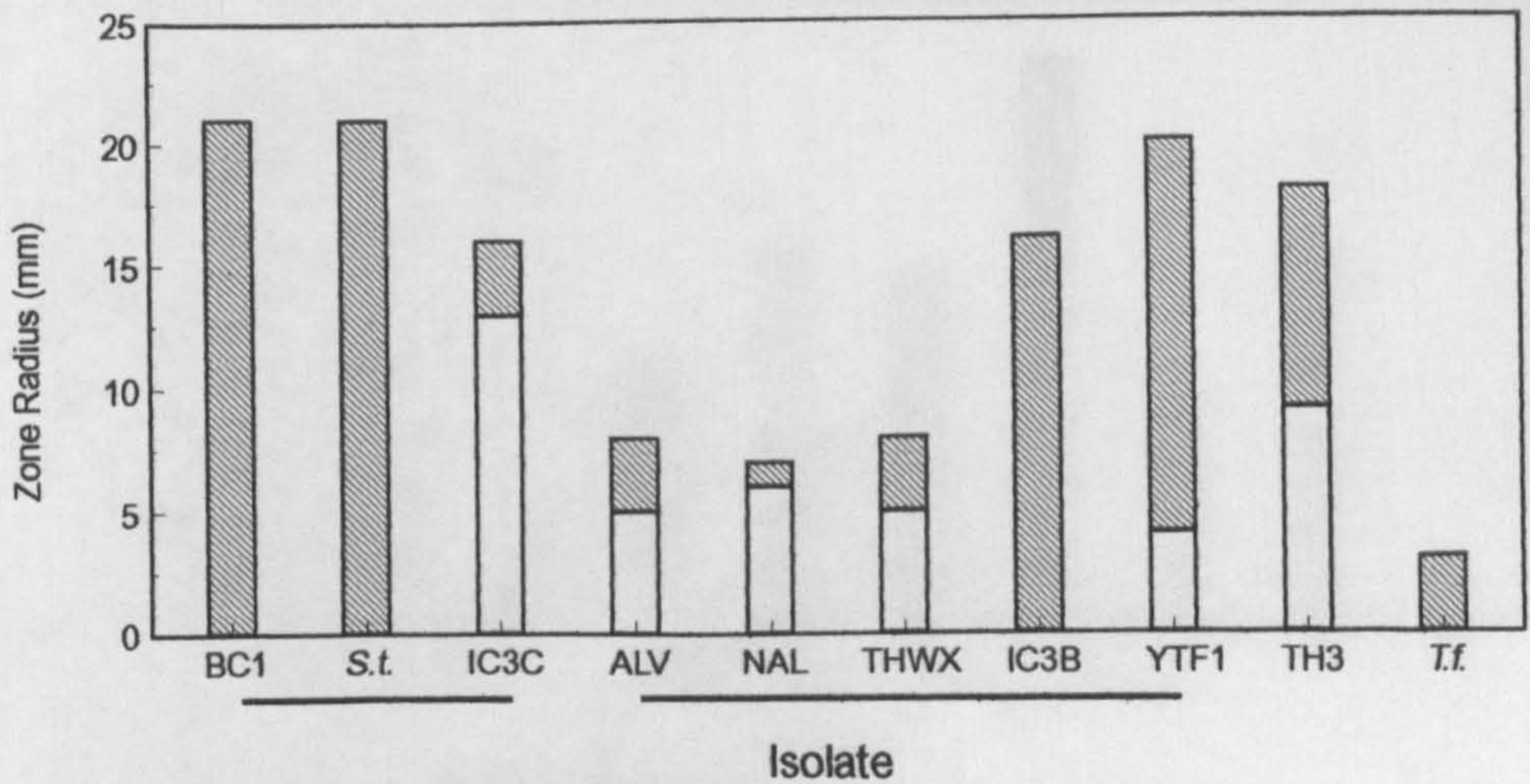
3.17 d: Fusidic Acid (10 µg)



3.17 e: Novobiocin (5 µg)



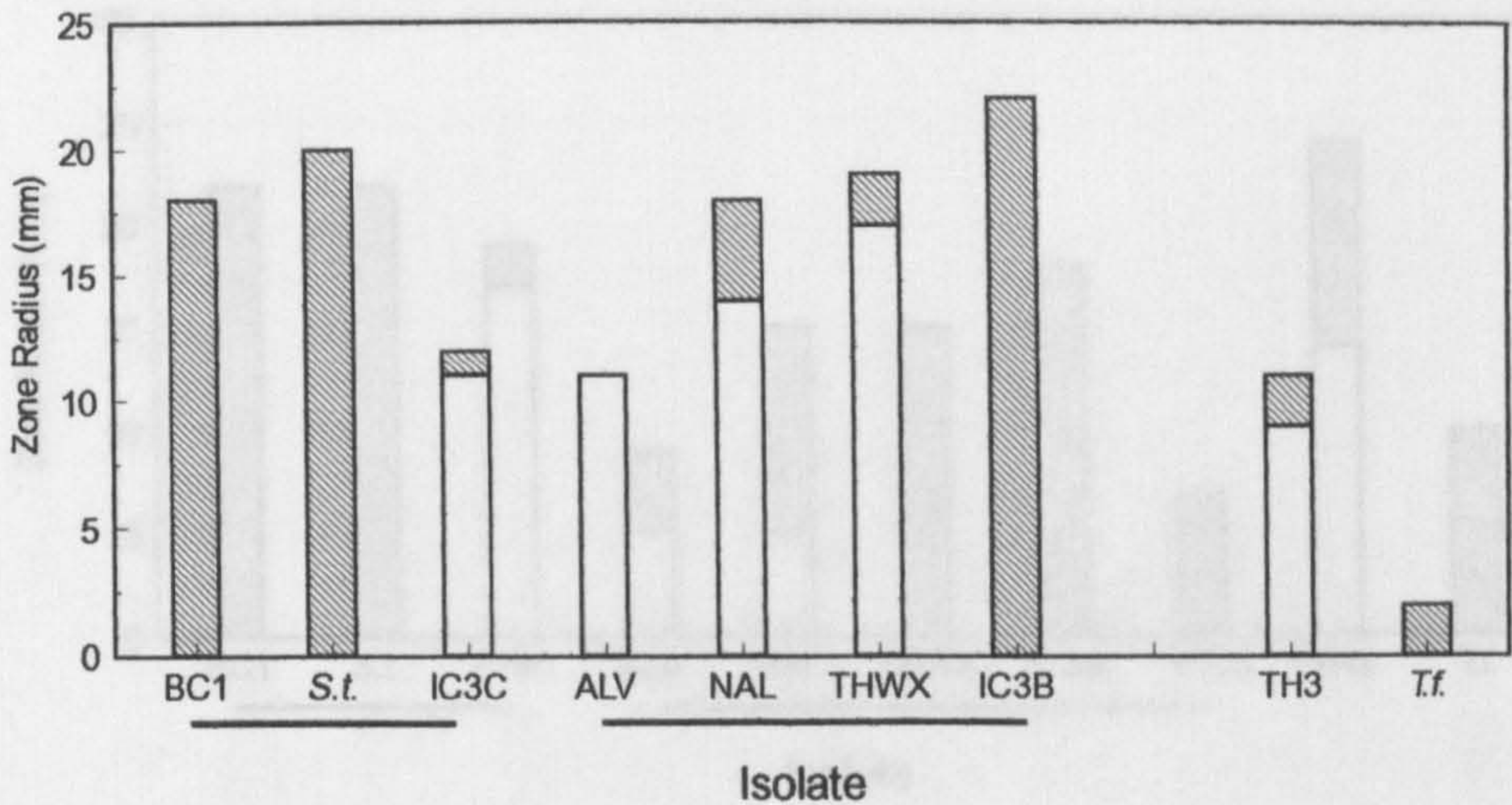
3.17 f: Tetracycline (25 µg)



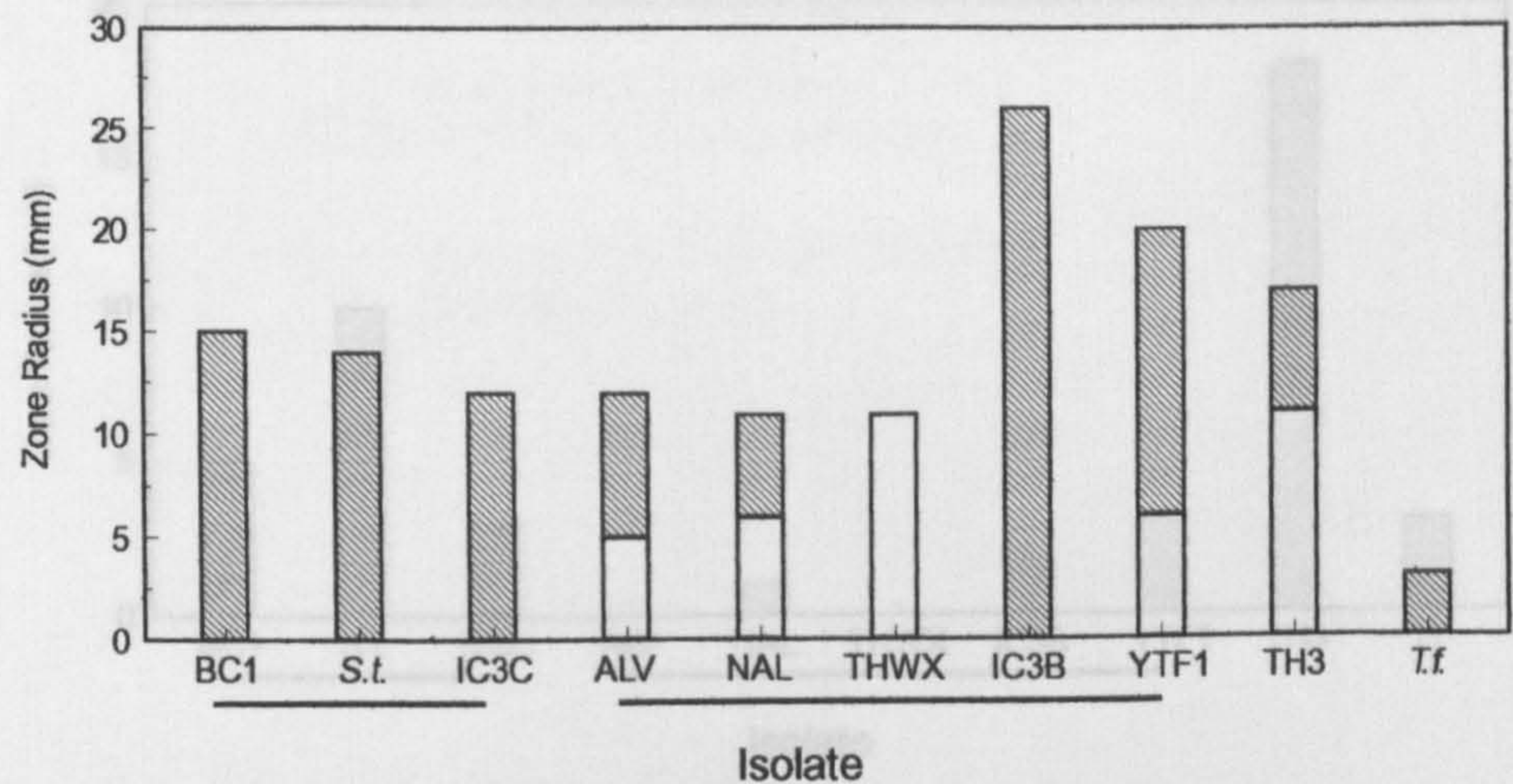
□ Zone of Growth Inhibition.
▨ Zone of Iron Oxidation Inhibition.

— Isolates from the same DNA:DNA hybridisation group.

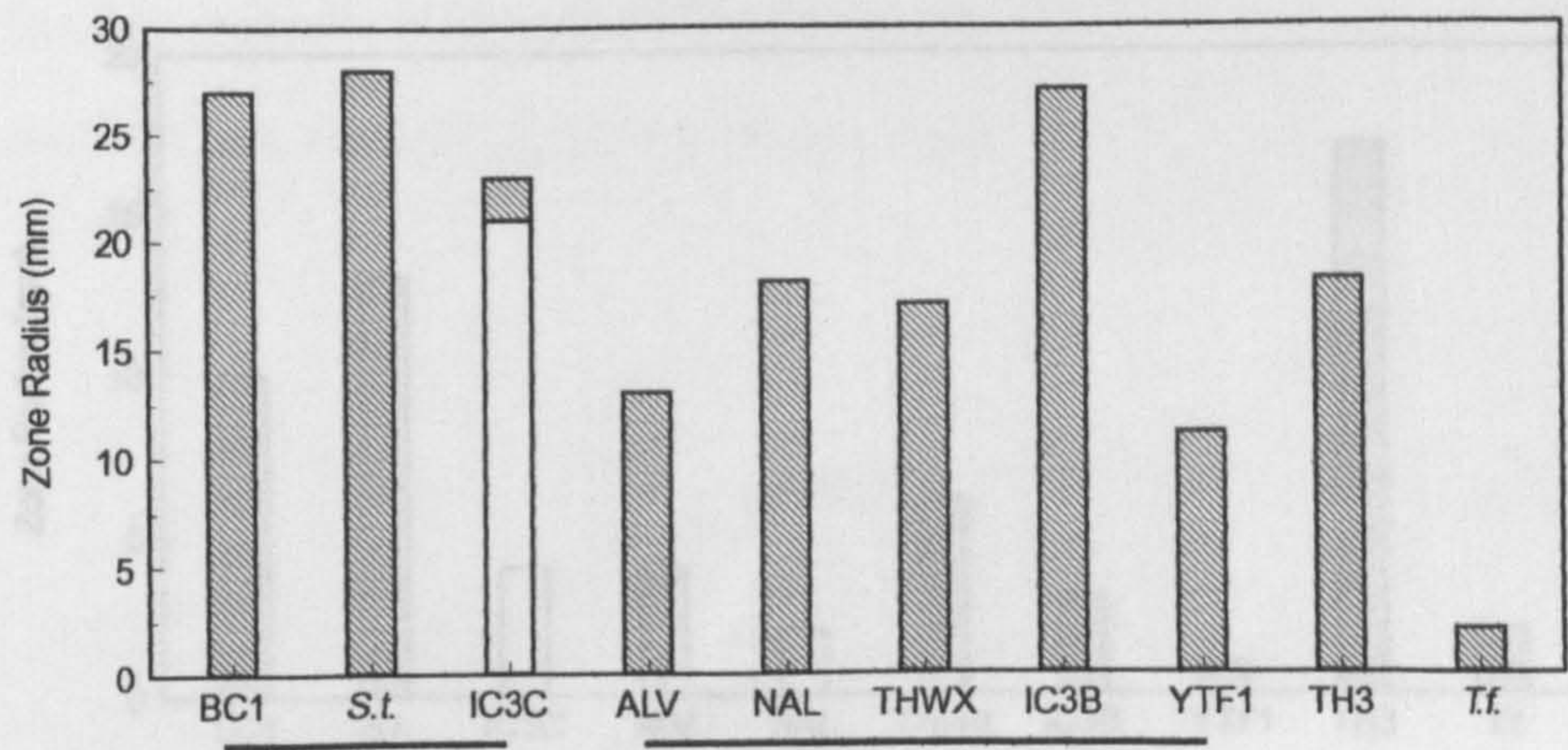
3.17 g: Thiostrepton (20 µg)



3.17 h: Methicillin (10 µg)



3.17 i: Mezlocillin (30 µg)



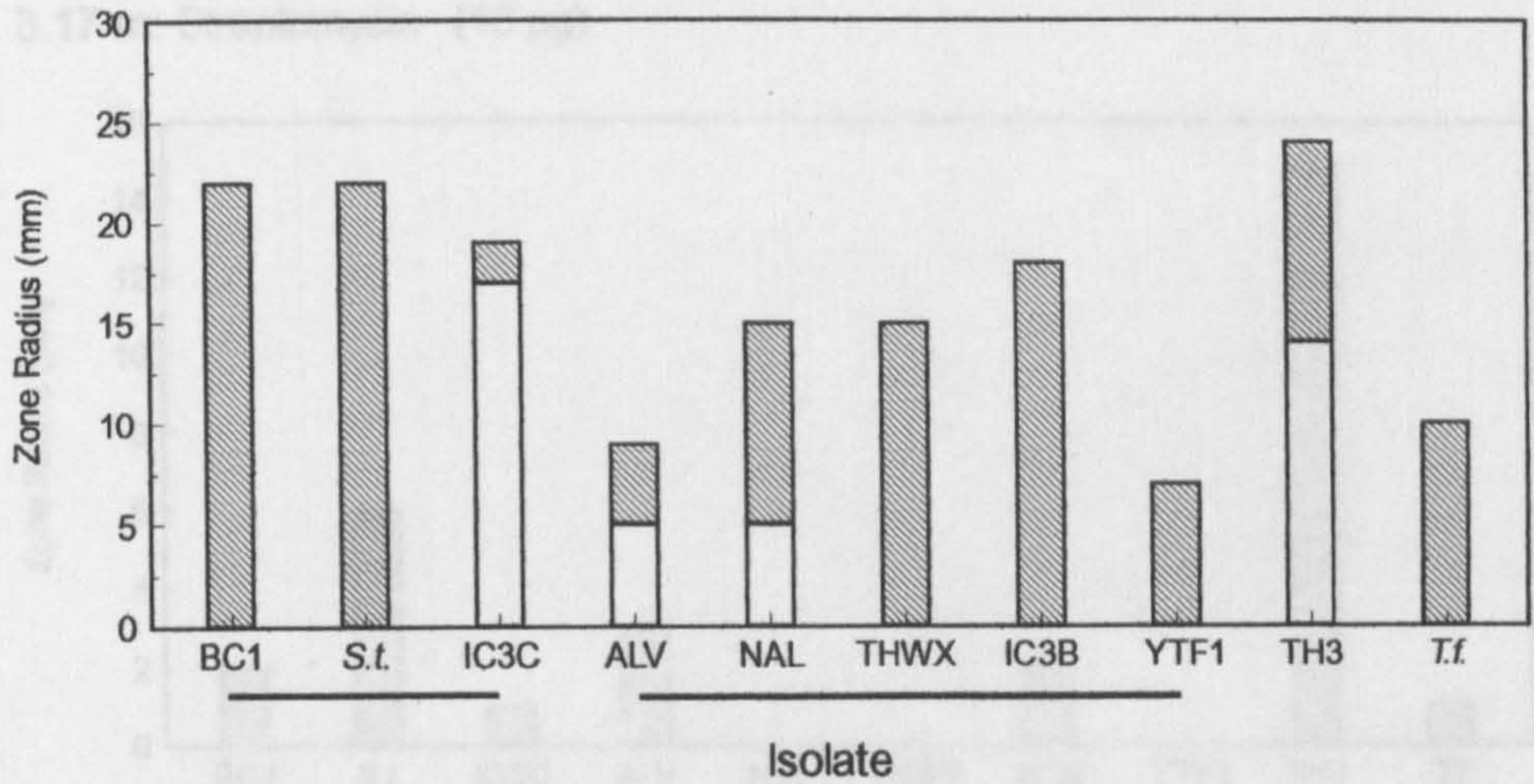
Isolate

□ Zone of Growth Inhibition.

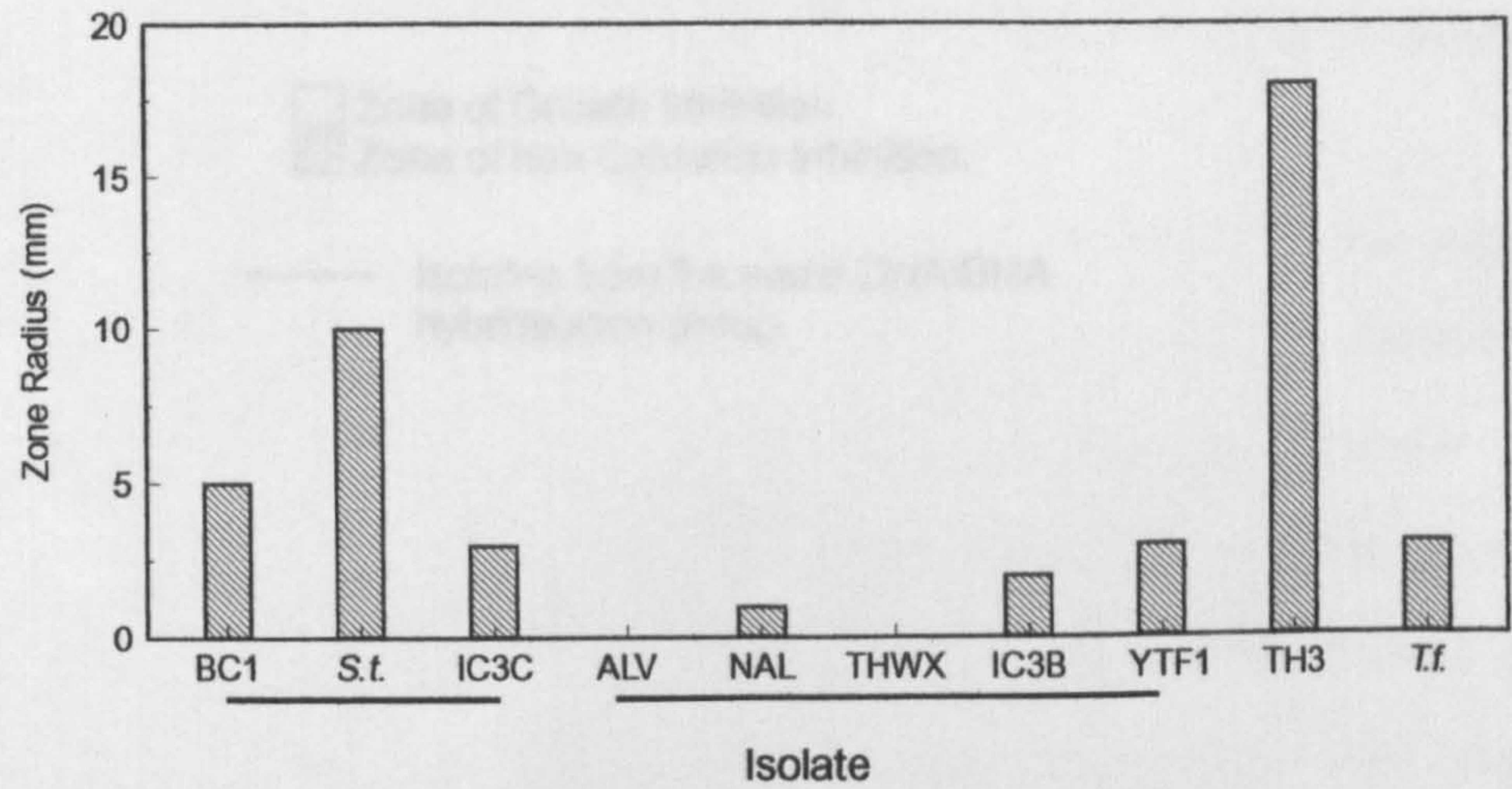
▨ Zone of Iron Oxidation Inhibition.

— Isolates from the same DNA:DNA hybridisation group.

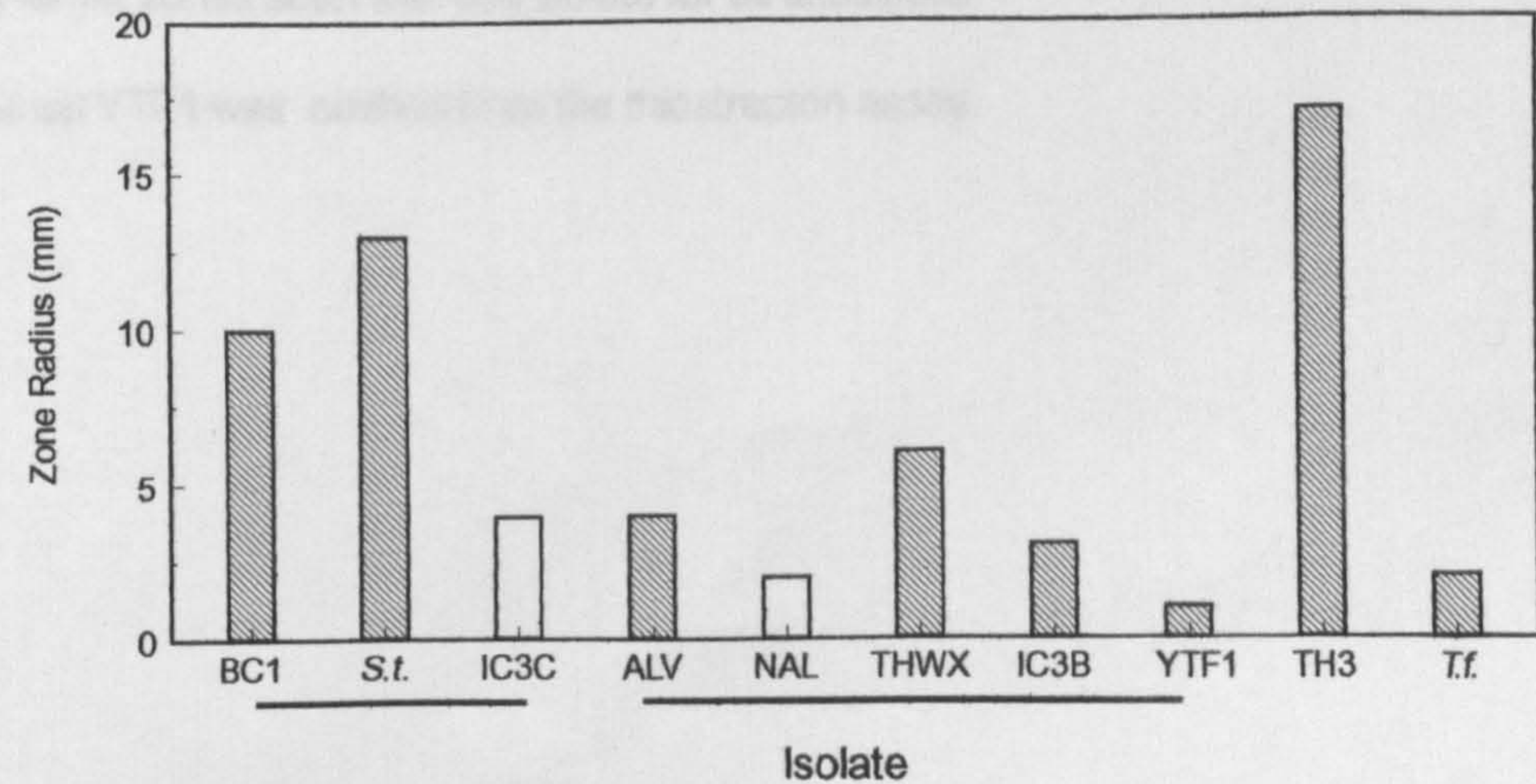
3.17 j: Ticarcillin (75 µg)



3.17 k: Amikacin (30 µg)



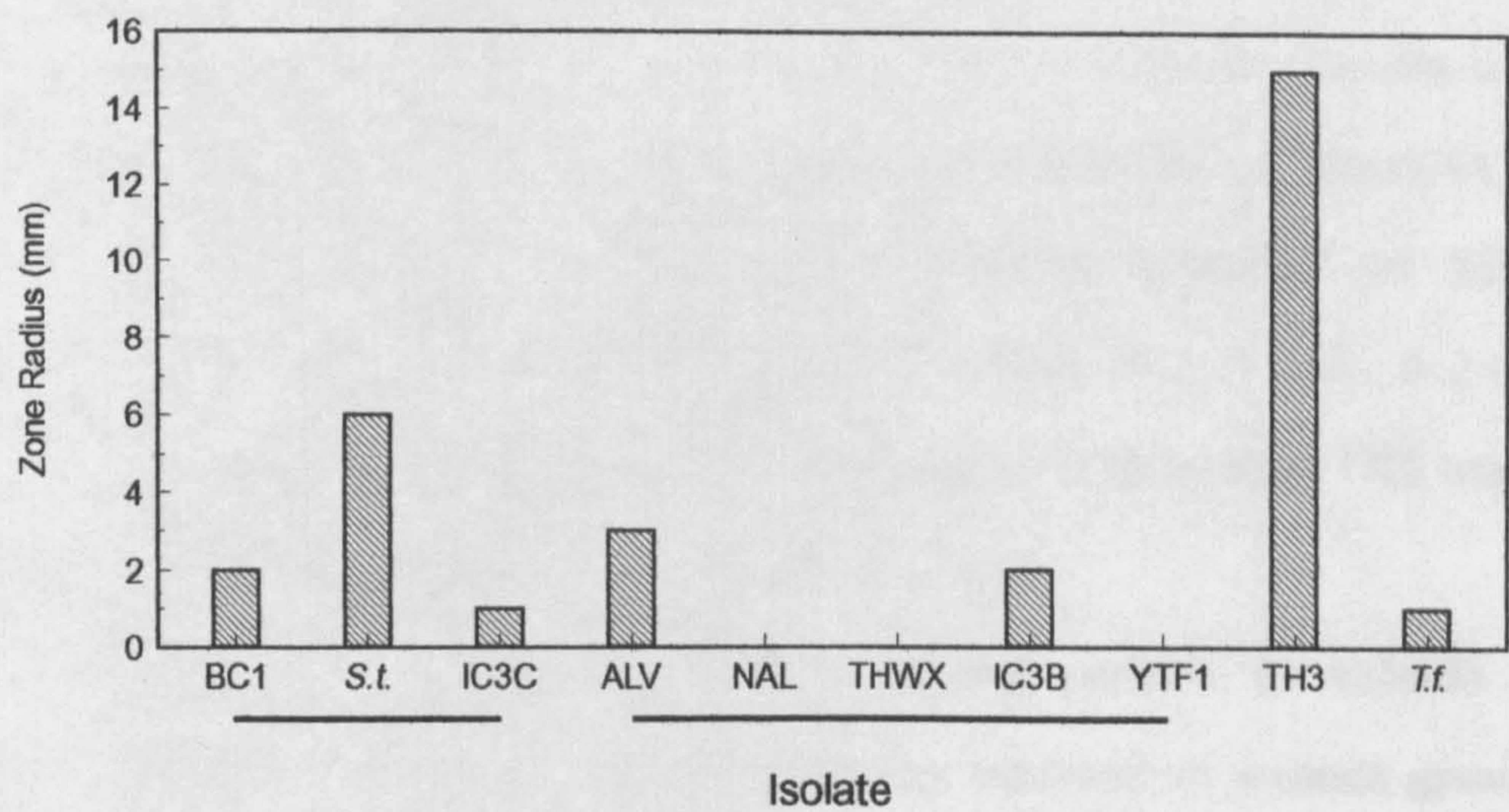
3.17 l: Gentamycin (10 µg)



□ Zone of Growth Inhibition.
▨ Zone of Iron Oxidation Inhibition.

— Isolates from the same DNA:DNA hybridisation group.

3.17 m: Streptomycin (10 µg)



□ Zone of Growth Inhibition.
▨ Zone of Iron Oxidation Inhibition.

— Isolates from the same DNA:DNA hybridisation group.

- Strain IC3B grew poorly compared to the other isolates, and this may have been reflected in the larger zones seen with this isolate for all antibiotics.
- Strain YTF1 was omitted from the thiostrepton assay.

Novobiocin gave a similar pattern to that seen with colistin sulphate (Fig. 3.17 e.). Strain TH3 was the most sensitive, then the *S. thermosulfidooxidans* hybridisation group organisms, and finally the NAL group organisms were the least sensitive (with the exception of strain ALV). *T. ferrooxidans* was more sensitive than the NAL group isolates to novobiocin, but less sensitive than all the other isolates.

Tetracycline and all the penicillin antibiotics produced no noticeable trends between the different DNA:DNA hybridisation groups (Fig. 3.17 f., h.-j.).

Thiostrepton was the only antibiotic tested to which strain TH3 was less sensitive than the other moderate thermophiles (Fig. 3.17 g.).

The aminoglycosides followed a similar pattern to colistin sulphate and novobiocin (Fig. 3.17 k.-m.). Strain TH3 was inhibited to a much greater extent than any of the other isolates, and the *S. thermosulfidooxidans* group appeared to be more sensitive than the NAL group.

Overall strain TH3 was more sensitive to inhibition by the range of antibiotics tested than the other two DNA:DNA hybridisation groups of moderately thermophilic iron-oxidizing bacteria. The aminoglycoside antibiotics in particular produced larger zones with strain TH3 than any other isolate. Strain TH3 was the most resistant of the moderate thermophiles to thiostrepton. The *S. thermosulfidooxidans* group, although generally not as sensitive as strain TH3, were more sensitive to the range of antibiotics used than the NAL group, with greater sensitivity to novobiocin and colistin sulphate in particular. All of the Gram-positive moderate thermophiles were more susceptible to the range of antibiotics tested here than the Gram-negative mesophile *T. ferrooxidans*.

The results show that, despite problems of antibiotic inactivation at low pH and high temperature, differences in the relative sensitivities of the three DNA:DNA hybridisation groups of moderately thermophilic iron-oxidizers to a range of antibiotics were seen during pour plate diffusion assays.

3.6.4. Growth of moderately thermophilic iron-oxidizing bacteria in the presence of metals.

The toxicity of metals is not only relevant because metal resistance genes are potentially selective markers, but also because high levels of certain metals, such as arsenic, are often present in mineral leaching environments. The toxic affects of metals can vary according to the amount of organic material present (which binds metals), amount of precipitation, and ionic interactions. The pH can also have a considerable affect on metal availability and toxicity. Generally at low pH metals are more likely to exist as free cations. Just as was the case with the antibiotic assays comparison of the toxicity of metals on different isolates is possible providing that identical conditions are used during the growth and testing of the isolates.

Pour plate assays identical to those for antibiotics were used. 20 μ l of metal solution was applied to each assay filter disc (Whatman). The metal solutions used were copper sulphate (1 M), uranyl sulphate (100 mM), nickel sulphate (1 M), sodium arsenate (10, 100, 200 mM), sodium arsenite (10, 100, 200 mM). Plates were incubated and zones of inhibition measured, as for antibiotic assay plates.

Nickel sulphate produced no zones of inhibition with any of the organisms, and in several cases iron oxidation was enhanced around the filter. Sodium arsenite produced inconsistent results due to rapid precipitation of iron/arsenite compounds around the filter immediately upon placing the filter on the plate. Figure 3.18 shows the results obtained with the other metals.

Strain ALV was previously found to be more sensitive to copper and strain TH3 less sensitive than strain BC1 (Norris, 1989a). This was confirmed by the results obtained here (Fig. 3.18 a.). The *S. thermosulfidooxidans* group had a greater resistance to copper than the NAL group, but not as great as strain TH3.

Fig. 3.18 b. indicates that strain TH3 was more sensitive to uranyl sulphate than the *S. thermosulfidooxidans* group. This contrasts to the results obtained previously with strain TH1, a *S. thermosulfidooxidans* group organism (Norris, 1989a). The

S. thermosulfidooxidans group isolates were less sensitive to uranyl sulphate than any other isolate, with the exception of strain THWX.

Only slight differences in the sensitivity of the three groups to sodium arsenate were seen (Fig. 3.18 c.). The *S. thermosulfidooxidans* group was more inhibited by 200 mM sodium arsenate than the other groups.

Sensitivities of the three DNA:DNA hybridisation groups to particular metals indicates further differences between the groups. Strain TH3 was the least sensitive to copper (as may have been expected for a strain isolated from a copper mine), whereas the NAL group were the most sensitive. The *S. thermosulfidooxidans* group were the least sensitive to 100 mM uranyl sulphate, but the most sensitive to 200 mM sodium arsenate.

Figure 3.18: Metal disc diffusion assays.

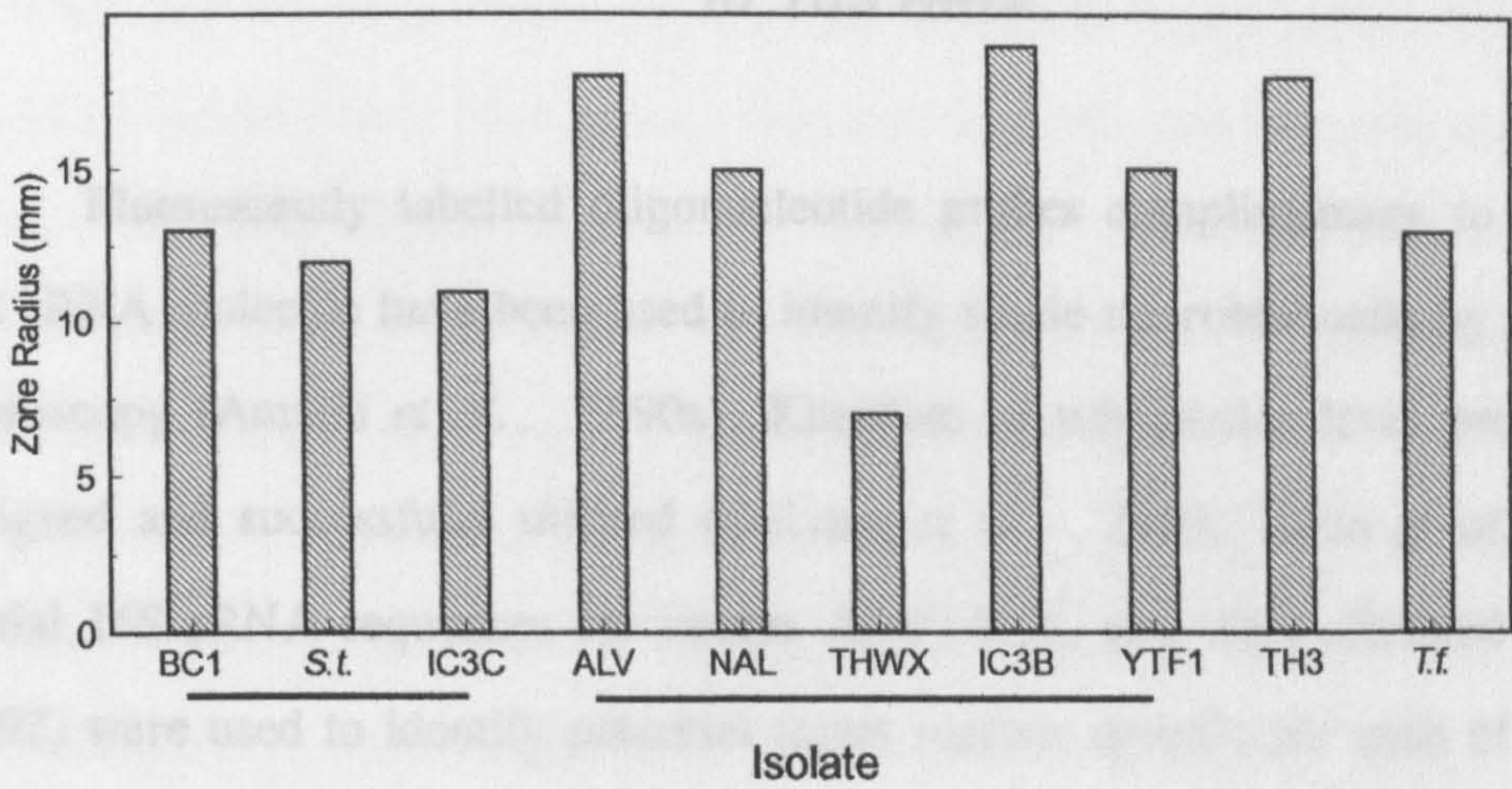
The three graphs illustrate the sizes of zones of iron oxidation inhibition (mm) produced by filter discs (Whatman) impregnated with 20 μ l metal solution. The radius of the zone was measured from the edge of the disc to the periphery of the zone. Iron oxidation was clearly visible by the production of ferric hydroxide precipitates within the medium.

All zones were measured after 7 days incubation. All moderate thermophile plates were incubated at 48 °C, whilst *Thiobacillus ferrooxidans* plates were incubated at 30 °C.

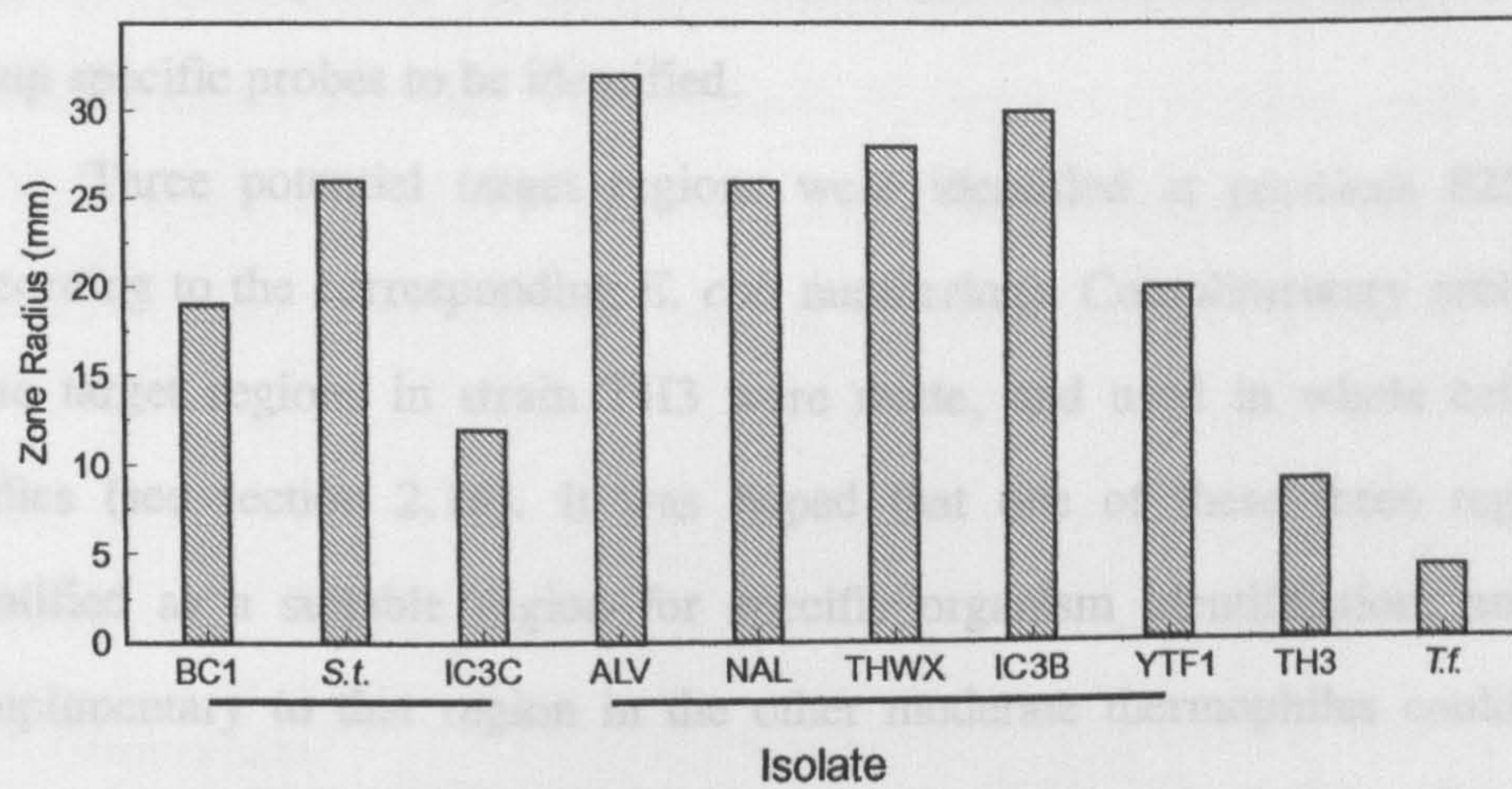
The isolates tested were as follows:

BC1	Moderate thermophile strain BC1.
<i>S.t.</i>	Moderate thermophile <i>Sulfobacillus thermosulfidooxidans</i> .
IC3C	Moderate thermophile strain IC3C.
ALV	Moderate thermophile strain ALV.
NAL	Moderate thermophile strain NAL.
THWX	Moderate thermophile strain THWX.
IC3B	Moderate thermophile strain IC3B.
YTF	Moderate thermophile strain YTF.
TH3	Moderate thermophile strain TH3.
<i>T.f.</i>	Mesophile <i>Thiobacillus ferrooxidans</i> .

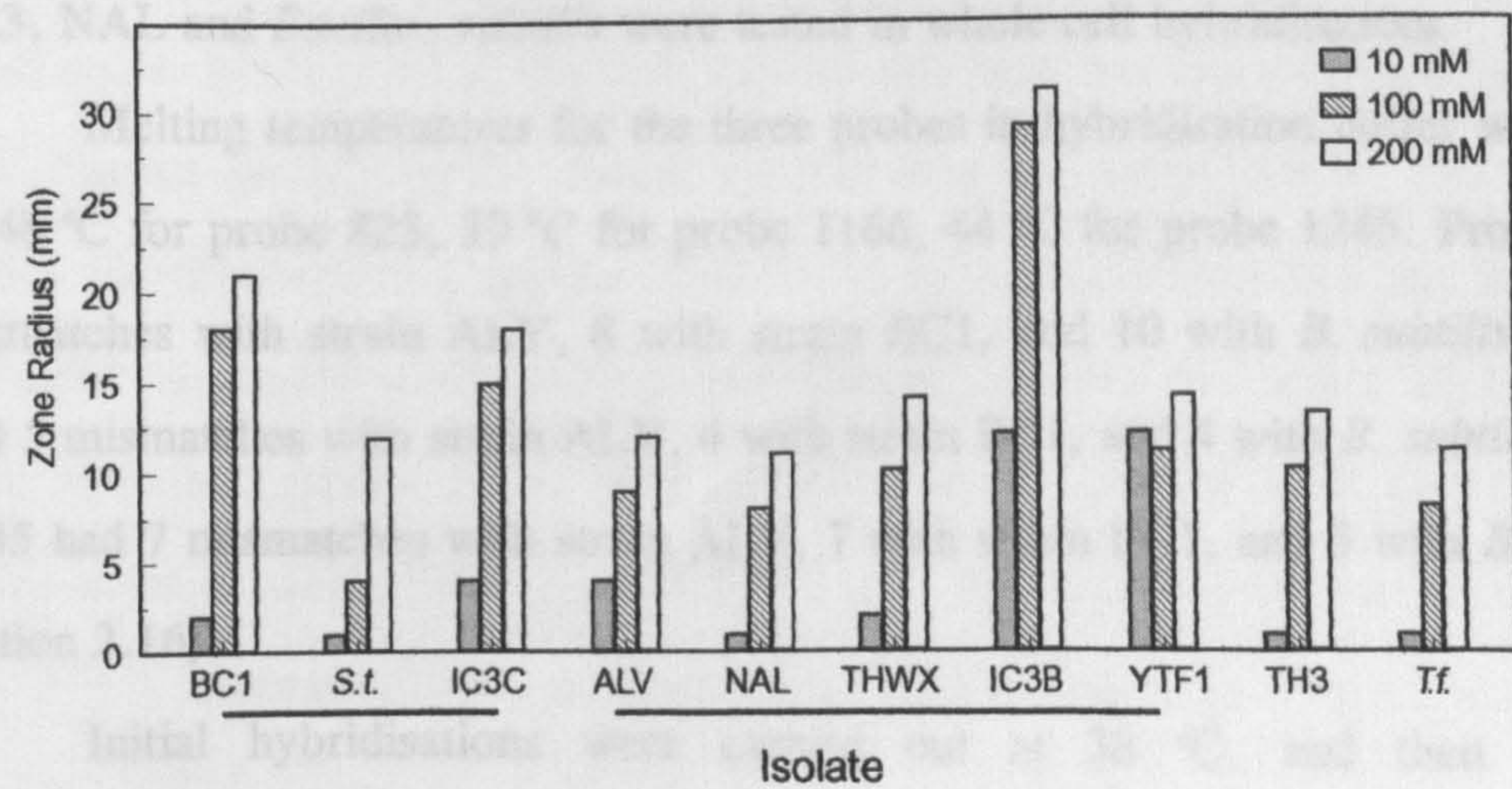
3.18 a: Uranyl sulphate (100 mM).



3.18 b: Copper Sulphate (1 M)



3.18 c: Sodium arsenate



Isolates from the same DNA:DNA hybridisation group.

3.7: Use of fluorescently labelled oligonucleotide probes complimentary to 16S rRNA.

Fluorescently labelled oligonucleotide probes complimentary to regions of the 16S rRNA molecule have been used to identify single microbial cells by epifluorescence microscopy (Amann *et al.*, 1990a). Kingdom to sub-species level probes have been designed and successfully utilised (DeLong *et al.*, 1989; Hahn *et al.*, 1993). The partial 16S rRNA sequences for strains ALV, BC1, and TH3 obtained by Lane *et al* (1992) were used to identify potential target regions specific for each of these isolates. These isolates are representatives from the three DNA:DNA hybridisation groups of moderate thermophiles, and so it was hoped that sequence differences would also enable group specific probes to be identified.

Three potential target regions were identified at positions 825, 1166, 1245 (according to the corresponding *E. coli* numbering). Complimentary probes specific for these target regions in strain TH3 were made, and used in whole cell hybridisation studies (see section 2.16). It was hoped that one of these three regions could be identified as a suitable region for specific organism identification, and then probes complimentary to that region in the other moderate thermophiles could be made. To ensure that sufficient permeabilisation of cells had occurred the universal eubacterial probe, Eub338 (Amann *et al.*, 1990b), was used. Mixtures and pure cultures of strains TH3, NAL and *Bacillus subtilis* were tested in whole cell hybridisations.

Melting temperatures for the three probes in hybridisation buffer were estimated as 48 °C for probe 825, 39 °C for probe 1166, 44 °C for probe 1245. Probe 825 had 9 mismatches with strain ALV, 8 with strain BC1, and 10 with *B. subtilis*; probe 1166 had 5 mismatches with strain ALV, 4 with strain BC1, and 4 with *B. subtilis*; and probe 1245 had 7 mismatches with strain ALV, 7 with strain BC1, and 5 with *B. subtilis* (see section 2.16).

Initial hybridisations were carried out at 38 °C, and then hybridisation temperature was increased in 2 °C increments until specific binding with strain TH3

cells occurred. All cells were clearly visible with the eubacterial probe up to a hybridisation temperature of 42 °C, indicating that permeabilisation of the cells had occurred, and the cells contained sufficient ribosome target molecules. Unfortunately specificity of the three TH3 probes was not obtained. At low temperatures the TH3 probes were still clearly binding to strain NAL and *B. subtilis* cells, at higher temperatures binding did not occur with any cells. Hybridisation conditions that allowed only specific binding to strain TH3 cells were not obtained.

No clear reasons for these results were evident. Some of the most common problems encountered with this technique are listed by Amann *et al* (1992). False negative results can occur because of limited permeabilisation of cells, or a low cellular ribosome content. The clear signal obtained with the universal Eubacterial probe suggests that neither of these factors was a problem. False positives can result from auto-fluorescence of cells, or non-specific binding of the probe. Auto-fluorescence of the bacteria did not occur. Therefore, the only possible explanation for the results obtained was that the probes were binding non-specifically.

3.8: Discussion.

The molecular, physiological and morphological studies of the moderately thermophilic iron-oxidizing bacteria carried out here has enabled their recognition as three distinct groups which should receive separate genus status. The conventional method for delineation of species DNA:DNA hybridisation showed that the first genus contained the isolate *Sulfobacillus thermosulfidooxidans* and strain BC1, the second genus contained strains ALV, NAL, IC2B, and N, and the third genus contained a single isolate, strain TH3 (Fig. 3.2). Using the classification scheme of Johnson (Fig. 3.3) *S. thermosulfidooxidans* and strain BC1 are varieties within a subspecies, and

strains ALV, NAL, IC2B, and N are also varieties within a subspecies, but of a different genus to *S. thermosulfidooxidans*.

This separation of the isolates into three main groups was supported by comparative protein electrophoretic methods. This type of classification method has proved to be useful in the separation of a variety of different isolates of bacteria and yeast (Dicks and van Vuuren, 1987; Pot *et al.*, 1989; Vauterin *et al.*, 1991). Although the use of this method was hampered here by the lack of computer facilities for analysis of large numbers of patterns, the close agreement of the results with those of the DNA:DNA hybridisation studies indicates the validity of the method. The establishment of pattern databases and computer-aided analysis should enable the technique to become a powerful identification tool (Vauterin and Vauterin, 1992).

The analysis of DNA base composition confirmed the results of Harrison (1986), and provided another method of separating the three genera. The *S. thermosulfidooxidans* group organisms have G+C values between 47-50 mol%, the second DNA:DNA hybridisation group have G+C contents between 54-57 mol%, and strain TH3 has a G+C content of 67-68 mol%. This separation of strain TH3 into the high G+C sub-division of Gram-positive bacteria has been confirmed by the 16S rRNA analysis work of Lane *et al.* (1992).

The morphological and physiological studies carried out highlighted other differences between isolates which further characterise the three DNA:DNA hybridisation groups. These morphological and physiological differences could not be used as the sole basis of isolate classification, but they do enable preliminary identifications to be made. For example, poor heterotrophic growth on yeast extract would suggest a member of the *S. thermosulfidooxidans* group, whereas good autotrophic growth on sulphur would suggest a NAL group isolate.

On the basis of these studies all of the isolates available to us within this laboratory have now been placed within this grouping scheme. The information obtained is thought to be sufficient to enable the naming of the major groups and isolates, although ideally 16S rRNA analysis still needs to be carried out.

A recent paper describing the sequence analysis of 16S rRNA from *S. thermosulfidooxidans* (Tourova *et al.*, 1994) indicated a close relationship to the genus *Alicyclobacillus*, and not moderate thermophile strain BC1, in contradiction to all the work presented here. This difference in the classification of *S. thermosulfidooxidans* suggests that either Tourova *et al.* (1994) or this laboratory has used the wrong organism in their studies. One possibility for this disagreement is that the wrong sequence for strain BC1 was used by Tourova *et al.* (1994) in their phylogenetic tree. However, the partial 16S rRNA sequence of moderate thermophile strain BC1 used by Tourova *et al.* (1994) was that sequenced by Lane *et al.* (1992), and this has been confirmed as that of strain BC1 within this laboratory (S. Waterhouse, personal communication).

The second possibility is that a culture became contaminated prior to DNA isolation. In the sequence analysis of Tourova *et al.* (1994), *S. thermosulfidooxidans* was grown heterotrophically, under which conditions an acidophilic heterotroph would have a faster doubling time than *S. thermosulfidooxidans*. Therefore, unless careful microscopic analysis was carried out, a contaminant could easily have been cultured. Furthermore *Alicyclobacillus* species are acidophilic, thermophilic, heterotrophic Gram-positive organisms that grow between pH 2.0-6.0, and 40-70 °C (Wisotzkey *et al.*, 1992), suggesting that contamination of a heterotrophically grown *S. thermosulfidooxidans* culture could occur. It is therefore believed that the similarity in the 16S rRNA sequences of *S. thermosulfidooxidans* and the genus *Alicyclobacillus*, suggested by Tourova *et al.* (1994), is a result of the sequencing of the 16S rRNA from an *Alicyclobacillus* contaminant, and that *S. thermosulfidooxidans* and strain BC1 are closely related, as suggested by all the studies carried out in this laboratory.

This grouping scheme does not encompass all moderately thermophilic iron-oxidizing bacteria. Indeed at least one isolate (LM2) has been described that appeared unrelated to any of the isolates described here (Norris and Barr, 1985). This isolate has since been lost from our culture collection, making classification impossible. However, its isolation indicates that other moderately thermophilic iron-oxidizers do exist which

do not fit in this grouping scheme. Nevertheless, this scheme most probably covers the main isolates being used at present in various laboratories around the world.

Initial attempts to utilise fluorescent oligonucleotide probes specific to 16S rRNA failed. The lack of specificity did not appear to be a result of limited permeabilisation, as has been encountered with other Gram-positive organisms (Amann *et al.*, 1992). Low cellular ribosome levels, another common problem, did not seem to be the cause as the universal Eubacterial probe gave adequate signals. The most likely explanation was that the probes were binding to sequences within the cell non-specifically. This problem could be avoided in future by carrying out filter hybridisation experiments with RNA extracts. All potential probes could then be tested to ensure specificity to the target organism before whole cell hybridisations with fluorescent probes was undertaken. If a probe is specific in filter hybridisation experiments, but still produces a weak signal in whole cell hybridisations then multiple probing could be used to enhance the signal (Amann *et al.*, 1990b). Finally, the probe design here was based on the limited data available from partial 16S rRNA sequences (Lane *et al.*, 1992), as more sequence data becomes available identification of target sequences may be made easier.

Chapter 4:

Characterisation of an Icelandic

Mixed Culture.

4.1: Introduction.

The requirement of all the moderately thermophilic iron-oxidizers for levels of CO₂ above that in air during autotrophic growth (Norris, 1989b) is a potential industrial process complication and cost. Firstly, there is an incentive to operate bio-oxidation plants at higher solid concentrations to improve turnover (Bailey and Hansford, 1993). At these higher solid concentrations gas exchange becomes a limiting factor, making efficient CO₂ utilisation important. Secondly, the increased operating temperatures required for thermophiles increases the likelihood of CO₂ limitation (Boogerd *et al.*, 1990). This necessity for efficient CO₂ utilisation, which none of the moderate thermophiles previously studied possessed, provided a selective culture condition, growth under air, for the isolation of novel strains with improved commercial potential.

An enrichment culture was obtained from an Icelandic hot spring complex. This culture efficiently solubilised pyrite at 45 °C under air (Norris and Owen, 1993). Microscopy indicated that this culture contained a variety of acidophilic bacteria. Further leaching work with this mixed culture produced good rates of dissolution for a range of minerals at 48 °C, and growth of the culture up to 53-55 °C (see section 6.5). Consistently high rates of pyrite dissolution under air have been maintained by this culture for over three years.

The use of ferrous iron as substrate in place of pyrite was chosen to facilitate the isolation and characterisation of what was anticipated to be a key iron-oxidizing organism in this mixed culture, i.e. one with efficient CO₂ utilisation capacity.

4.2: Separation of isolates from the mixed culture.

The mixed culture was maintained for more than 35 sub-cultures of autotrophic growth on ferrous iron under air. During this time attempts were made to isolate novel moderate thermophiles.

Initially, simple plating techniques were used. The poor growth of many chemolithotrophs on solid media is known to cause difficulties in their isolation

(Johnson *et al.*, 1989). However, the use of an alternative gelling agent, Gelrite, allows colony formation with a variety of thermophilic organisms (Lin and Casidsa, 1984). The solid medium used consisted of salts at pH 2.2 and 0.4% (w/v) gelrite (see section 2.1). In addition growth substrates were added: 20 mM ferrous iron supplemented with 0.5 mM tetrathionate (for autotrophic growth), 20 mM ferrous iron supplemented with 0.02% (w/v) yeast extract (for chemolithoheterotrophic growth), and 0.02% (w/v) yeast extract (for heterotrophic growth). Colonies were picked, cultured in liquid and re-plated. All subsequent cultures were then characterised.

4.2.1: Isolation of culture ICC.

The key organism within this mixed culture was believed to be one that was capable of good autotrophic growth under air. After several attempts at isolating such an organism, most of which resulted in cultures that grew poorly, a culture was obtained that produced iron oxidation rates under air similar to those seen with the original mixed culture (Figure 4.1). This culture, designated ICC, had a maximal iron oxidation rate very similar to the original mixed culture (IC Mixed). This rate also compared favourably with strain BC1, which reached stationary phase after only 20% total iron oxidation. Strain BC1 was only capable of equivalent iron oxidation levels when CO₂ levels were enhanced (Figure 4.2). Iron oxidation by culture ICC remained relatively unaffected by this increase in CO₂ concentration.

Characterisation of the ICC culture was undertaken. Studies on CO₂ utilisation are described in the next chapter (see section 5.2), whilst all other studies are described here.

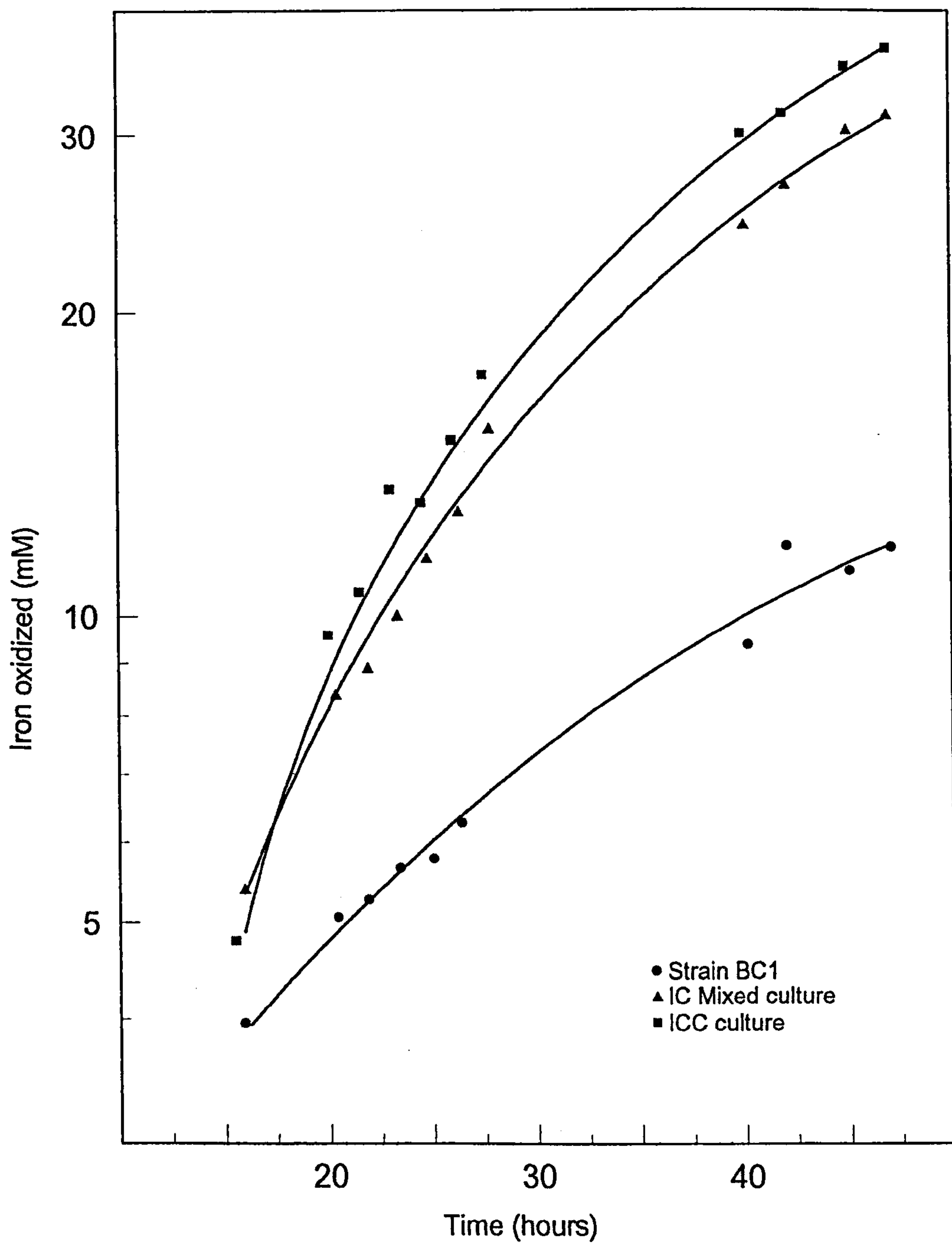


Figure 4.1: Iron oxidation during autotrophic growth of three moderately thermophilic cultures in pH 1.7 medium containing 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, under air, at 48 °C.

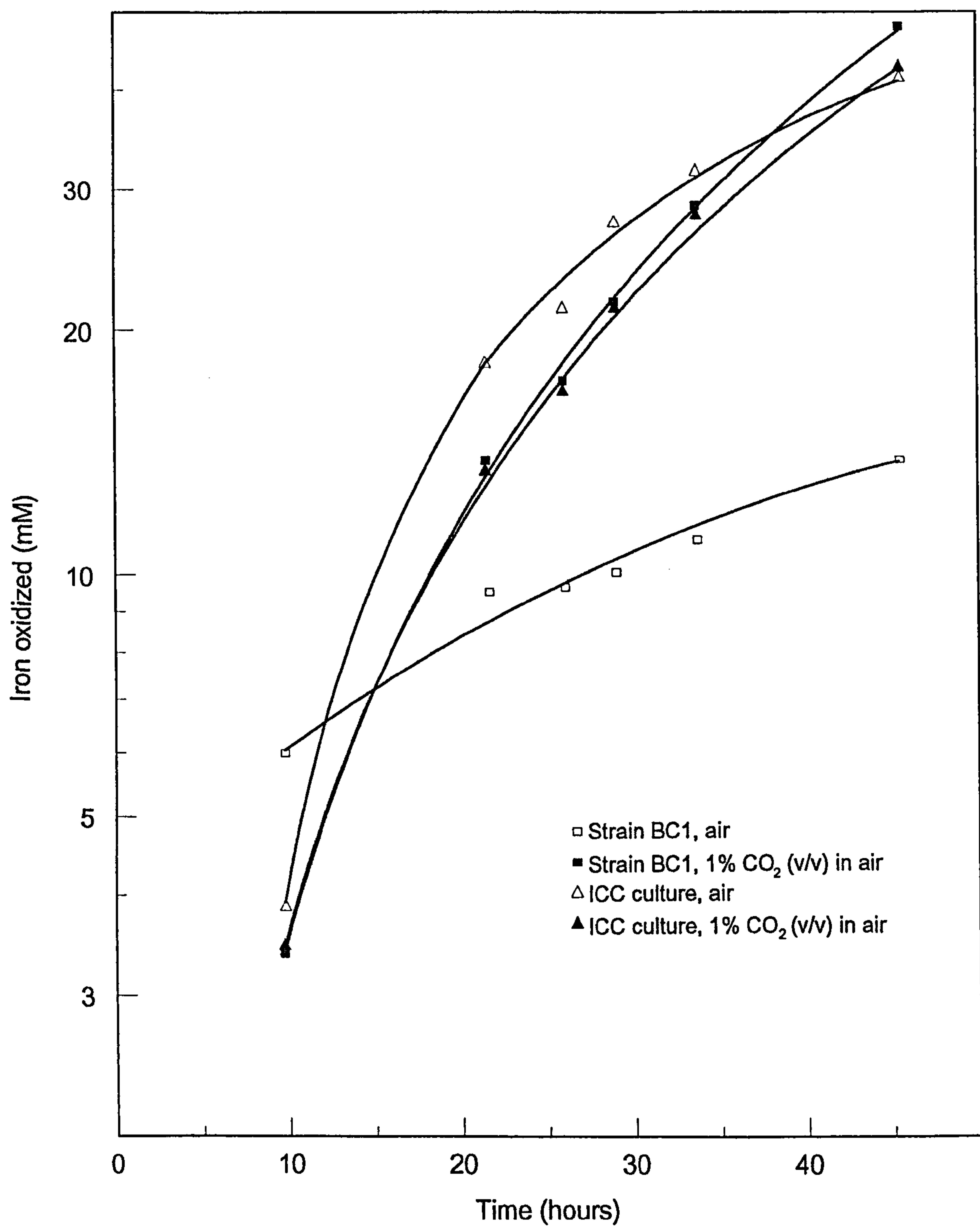


Figure 4.2: Iron oxidation during the autotrophic growth of moderate thermophile strain BC1, and culture ICC in the presence and absence of additional CO₂, at 48 °C. All cultures were grown in 1 l stirred reactors, gassed at 400 ml/min.

The analysis of protein electrophoretic patterns of culture ICC when grown chemolithoheterotrophically and autotrophically on iron indicated a high similarity to strain BC1. Isolation of organisms from heterotrophic and chemolithoheterotrophic plates also resulted in isolates with high homology to strain BC1. However, none of these isolates grew to a better extent under air than strain BC1. The reason for this failure of the isolated colonies to grow well under air, despite their electrophoretic protein patterns being identical to the ICC culture, was revealed by electron microscopy.

Thin section transmission electron microscopy of the ICC culture revealed that it was not a pure culture. Two bacterial types were clearly visible within this culture (Figure 4.3a. and b.). The first of these bacteria (labelled A, Fig. 4.3) had a typical "autotrophically-grown strain BC1-type" appearance with dimensions of $2.5\ \mu\text{m} \times 1.0\ \mu\text{m}$ (see Fig. 3.6 for comparison). The cells had a typical Gram-positive wall, an accumulation of negatively staining material at the poles of the cell, and formed endospores. The second cell type (labelled B, Fig. 4.3) was clearly distinguishable from the BC1-type organism, being much narrower ($0.3\ \mu\text{m}$), having inclusion bodies at the poles of the cell resembling polyphosphate granules (Shively, 1974), and with sporogenesis never being observed.

How these two organisms remained associated despite single colony isolation is not clear. Ferric iron precipitate is produced over colonies when grown on iron, making colony isolation difficult. A chance contamination by a spore or cell could, therefore, have occurred during colony selection.

The good autotrophic growth of the ICC culture indicates the importance of one or both of these organisms, no matter how these two cell types became associated. Several possibilities exist for the nature of the association of these two organisms. One organism could be an iron-oxidizer with a good CO_2 utilisation capacity, whilst the other could be a heterotroph that scavenges exogenous organic compounds produced by the iron-oxidizer. In this situation the second organism would be playing no role in the

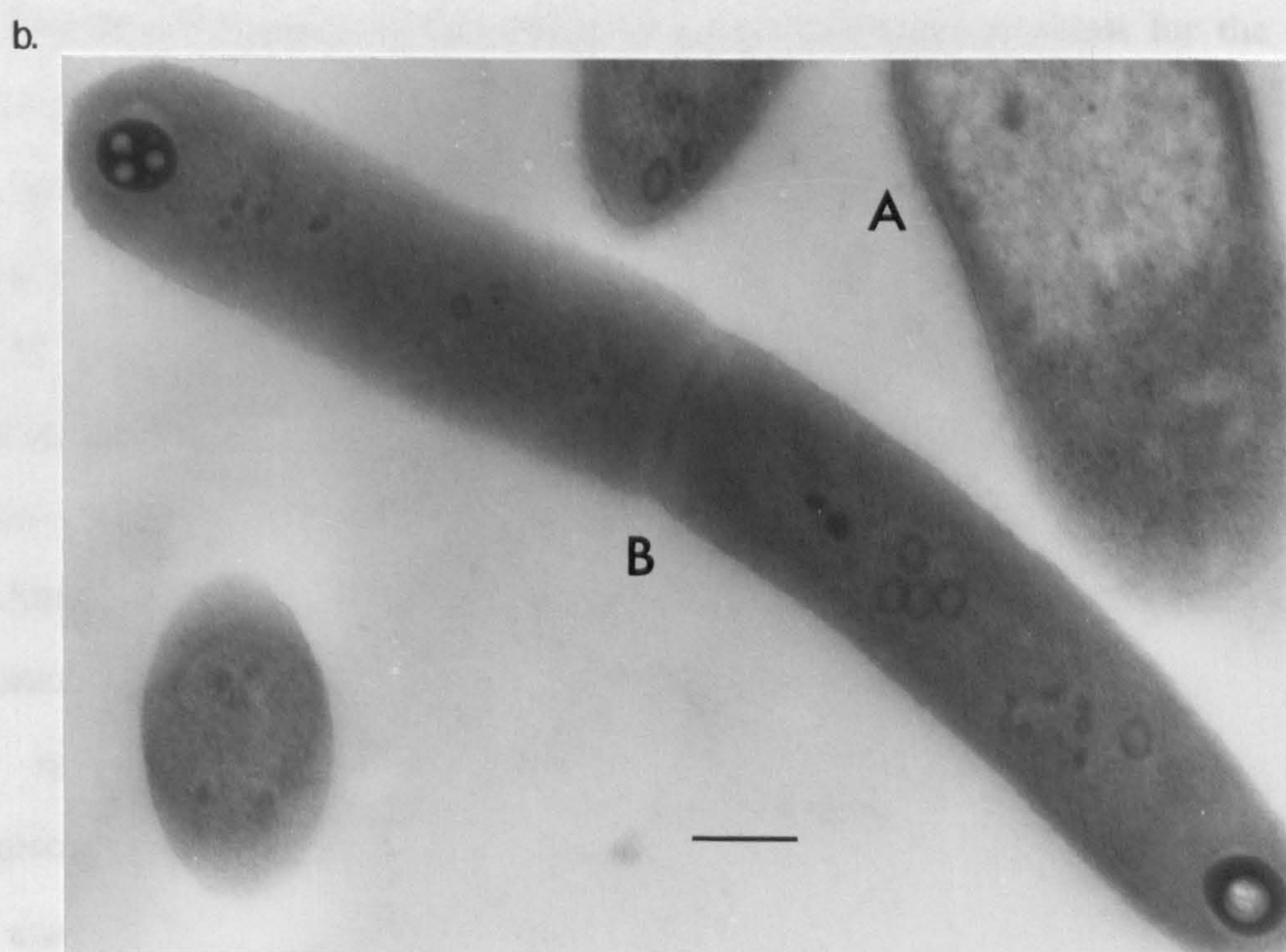
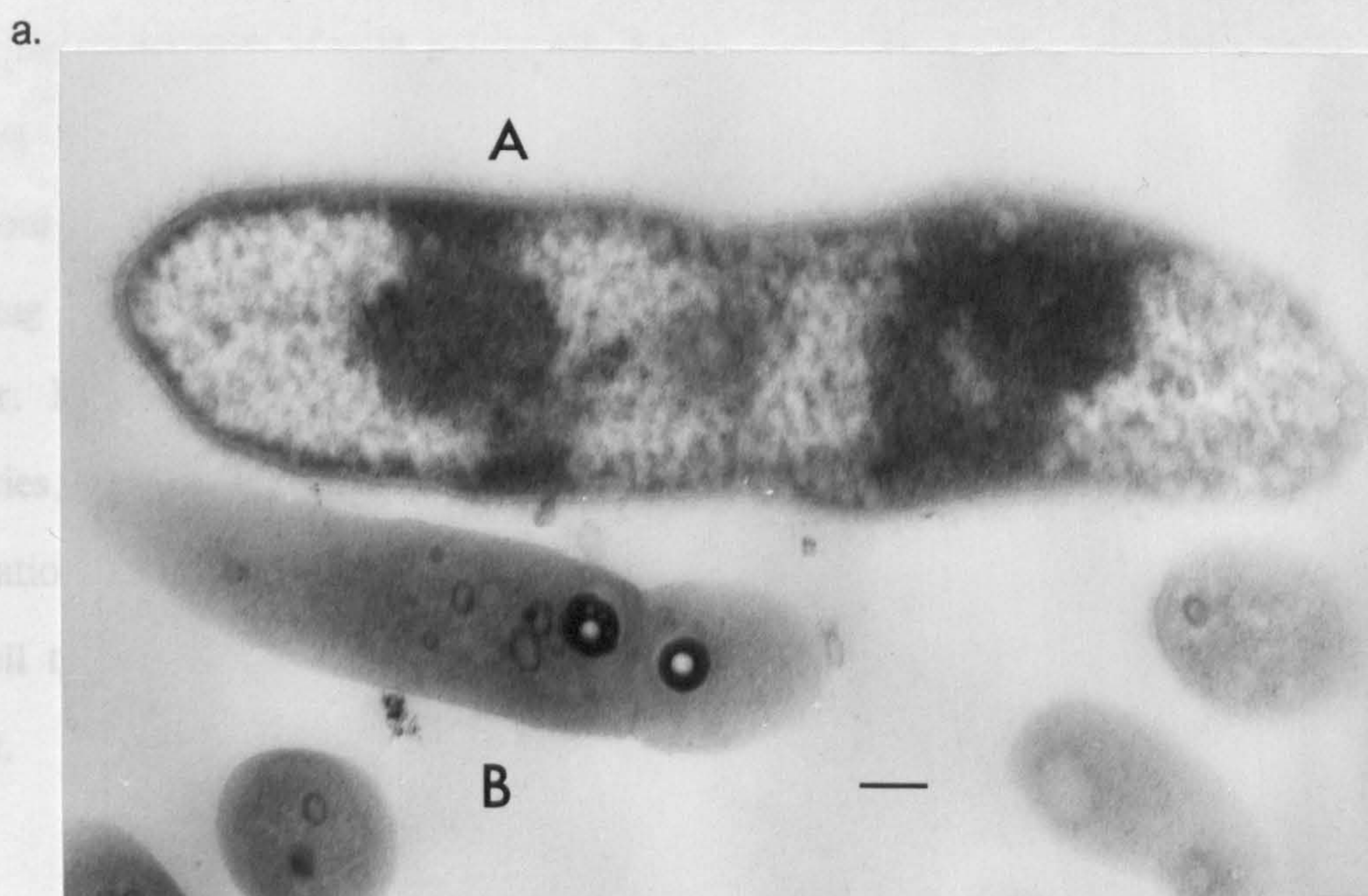


Figure 4.3: Thin section electron micrograph of culture ICC. Culture ICC was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, under air. Thin sections were stained with uranyl acetate prior to transmission electron microscope analysis. Two distinct cell types are visible, a BC1 type organism (labelled A), and a second thinner cell (labelled B). Bar represents 0.25 μm .

oxidation of minerals. The isolation of the BC1-type organisms from the mixture, and their poor autotrophic growth meant this theory was unlikely. An alternative theory was that the second organism was another iron-oxidizer. If this second iron-oxidizer possessed a good CO₂ utilisation capacity, then the BC1-type organism might be obtaining fixed carbon from it, and thus be surviving in a chemolithoheterotrophic manner. Finally, both organisms might be iron-oxidizers with good CO₂ utilisation capacities, but this would not explain the poor growth of the BC1-type cells. The second explanation, therefore, seemed the most plausible. Attempts were made to separate the two cell types from the mixed culture to elucidate which of these theories might be correct.

4.2.2: ICC enrichment cultures.

Various enrichment cultures were set up in an attempt to select for the novel organism within the ICC culture. The enrichments used included autotrophic growth at pH 1.7, and 48 °C on 50 mM ferrous iron in the absence of a reduced sulphur source, growth at pH 2.0 on 1 mM tetrathionate, and chemolithoheterotrophic growth at pH 1.7 and 48 °C on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract. A reduced sulphur source is a known requirement of all moderate thermophiles with the exception of strain ALV (Norris and Barr, 1985).

After six sub-cultures of the chemolithoheterotrophic enrichment, the culture was plated onto 20 mM ferrous iron supplemented with 0.02% (w/v) yeast extract. Single colony isolates were grown initially chemolithoheterotrophically, and then autotrophically under air. All these isolates grew poorly autotrophically under air. Protein analysis revealed high similarity to strain BC1 (Figure 4.4), suggesting the BC1-type organism within the culture was a good chemolithoheterotrophic iron-oxidizer, but a poor autotrophic iron-oxidizer.

The tetrathionate enrichment grew very poorly even with CO₂ supplement, taking three weeks for any visible growth. After several serial sub-cultures growth remained poor, but the culture had become dominated by a tiny rod-shaped cell.

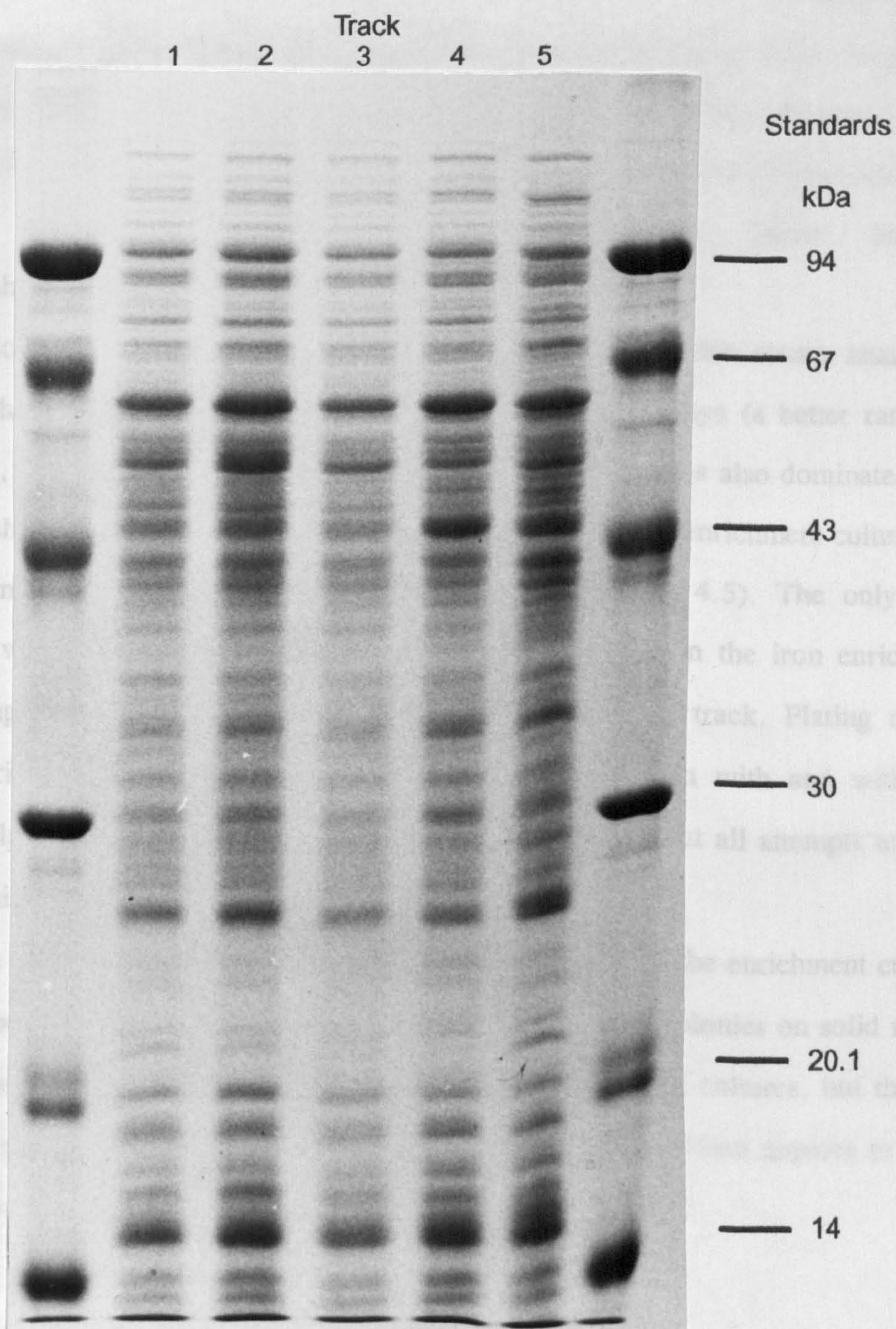


Figure 4.4: SDS-PAGE of BC1 type isolates from the ICC mixed culture (tracks 1-4). Whole cell protein samples from chemolithoheterotrophically grown isolates were run on a 10% (w/v) denaturing polyacrylamide gel, and stained with Coomassie blue. Strain BC1 (track 5) is included for comparative purposes.

The electrophoretic protein pattern of the enrichment culture showed low homology to both strains BC1 and ALV (Figure 4.5). Attempts to grow this culture on tetrathionate-containing plates failed to produce any colonies, but chemolithoheterotrophic growth on iron resulted in a number of colonies. Subsequent characterisation of these colonies indicated they were all BC1-type organisms. This suggested that the BC1 type organism was still present in this enrichment culture in sufficient numbers to enable colony formation on plates allowing chemolithoheterotrophic growth.

Autotrophic growth on ferrous iron without a reduced sulphur source resulted in a culture that oxidized about 40% of the ferrous iron in 5-7 days (a better rate than strain BC1, but much less than the ICC culture). This culture was also dominated by a small rod-shaped cell. The electrophoretic protein pattern of this enrichment culture was almost identical to the tetrathionate enrichment culture (Fig. 4.5). The only clear difference was a single protein band (approximately 35 kDa) in the iron enrichment track, compared to two bands in the tetrathionate-enrichment track. Plating of this second enrichment culture to give autotrophic growth on iron with and without a reduced sulphur source resulted in several pin prick colonies, but all attempts to grow these colonies in liquid medium failed.

The small rod-shaped cell that dominated at least two of the enrichment cultures could not be obtained in pure culture, because it did not form colonies on solid media. A BC1-type organism was isolated from all of these enrichment cultures, but this cell type grew poorly under air. The selection of the BC1-type organism appears to be an indication of its ability to form colonies on solid media.

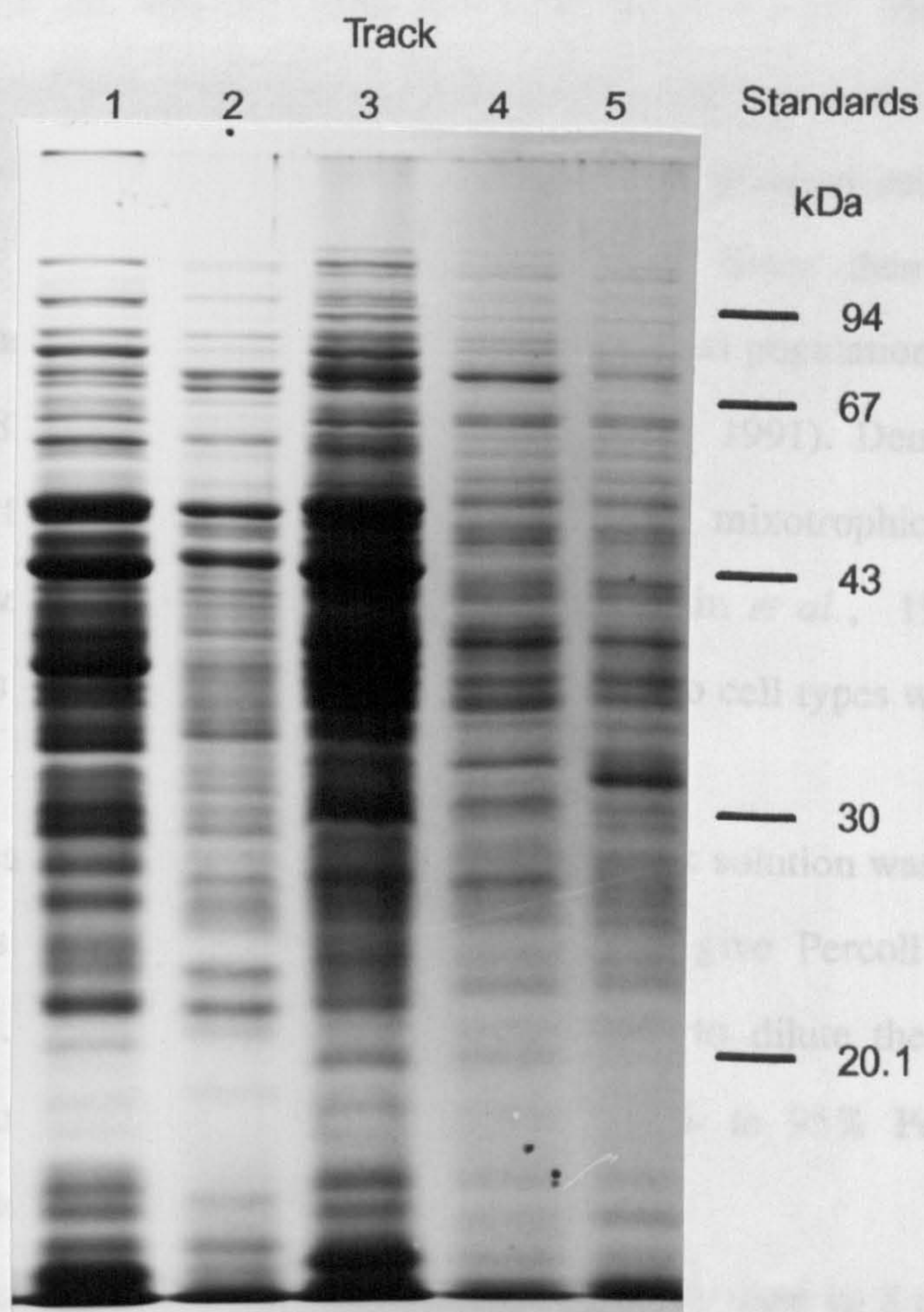


Figure 4.5: SDS-PAGE of ICC enrichment cultures. Culture ICC was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under air (track 3), 1 mM tetrathionate under 1% CO₂ (v/v) in air (track 4), and 50 mM ferrous iron under air, but without a reduced sulphur source (track 5). Whole cell protein samples were run on a 10% (w/v) denaturing polyacrylamide gel, and stained with Coomassie blue. Strains BC1 and ALV are included for comparative purposes (tracks 1 and 2 respectively).

4.2.3: Percoll buoyant density gradients.

Percoll is an inert solution of polyvinylpyrrolidone-coated silica beads, with a mean diameter of 17-30 nm, that are stable in the pH range 5.5-12. These beads can form self-generating gradients with high particle density and low osmolarity. Percoll was originally introduced as a non-toxic medium for density gradient centrifugation of mammalian cells and organelles (Pertoft *et al.*, 1978). Since then it has been successfully used for the separation of several different bacterial populations from mixed cultures (Scherer, 1983; Beaty *et al.*, 1987; Putzer *et al.*, 1991). Density gradients have also been used for the separation of the acidophilic mixotrophic contaminant *Thiobacillus acidophilus* from *T. ferrooxidans* cultures (Martin *et al.*, 1981). Density gradient separation was used here for the separation of the two cell types within the ICC mixed culture.

For the purposes of these experiments the Percoll stock solution was designated a 100% stock. Dilutions of this 100% stock were made to give Percoll gradients of varying concentrations. A 0.2 M glycine buffer was used to dilute the Percoll (see section 2.10). This buffer gave stable gradients from 50% to 95% Percoll, whilst maintaining the pH as low as possible (i.e. pH 5.5).

Initial experiments to optimise the Percoll concentration used an 8 ml gradient at concentrations of 50, 75, and 90%. Washed cell suspensions were loaded onto each gradient, and centrifugation was carried out (see section 2.10). In all the gradients a fraction of the cell suspension remained at the top of the tube. This fraction was believed to constitute aged and/or starved cells, and cell debris (Scherer, 1983).

The 50% gradient was too low a concentration, with all the cell suspension pelleting at the bottom of the tube, whereas no cells entered the 90% gradient. A diffuse area of cells was seen down the length of the 75% gradient.

A 75% gradient was clearly the approximate concentration for cell separation, and so gradients of 70-80% were set up to obtain clearer cell separation. Figure 4.6 shows some of the gradients between 70-75%. In the 70-72% gradients there was a diffuse band of cells 15-40 mm from the top of the gradient.

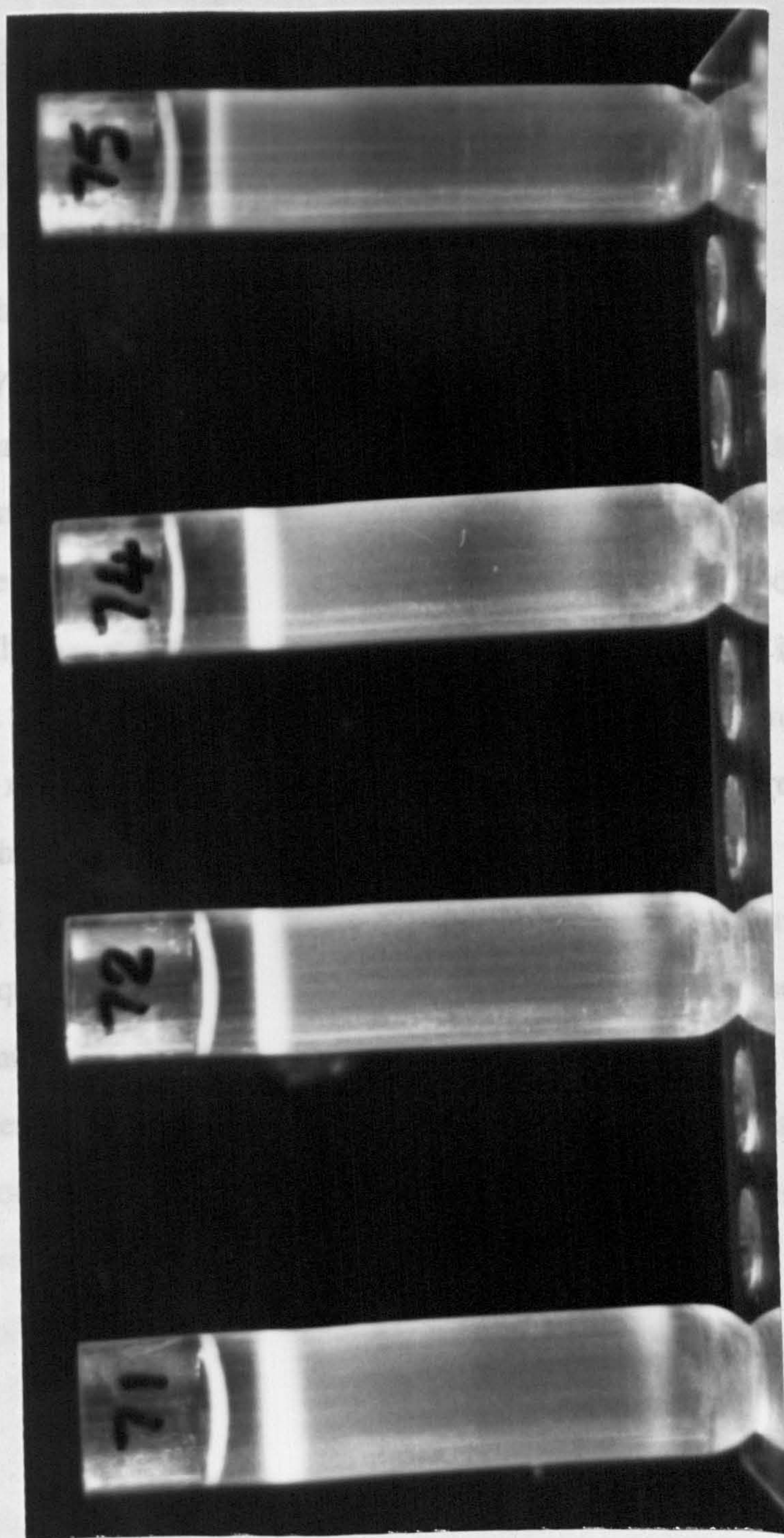


Figure 4.6: Position of culture ICC cell bands on four Percoll gradients. Autotrophically grown ICC cells were harvested and loaded onto a range of Percoll gradients at different concentrations. The position of cell bands are shown on 71, 72, 74, and 75% gradients (from left to right).

Below this diffuse band was a second sharp band, separated from the upper band by at least 10 mm. The 73-75% gradients resulted in the lower band becoming more diffuse, and the separation of the two bands becoming smaller. Gradient concentrations above 75% produced a single region of diffuse cells with no clear cell separation.

In all subsequent work 71% gradients were used for cell separations. The buoyant density distribution of a 71% gradient was determined with coloured marker beads of known density (Sigma Chemical Co.). The relative positions of the two cell bands from the ICC culture are indicated in Figure 4.7. Buoyant density values are for guidance only, as the marker beads are standardised for iso-osmotic Percoll in 0.15 M NaCl (Scherer, 1983). The upper diffuse cell band had a buoyant density in the range 1.08-1.09 g/ml, whereas the lower cell band had a buoyant density of 1.12 g/ml. Samples were carefully extracted from the two bands using a hypodermic needle, and viewed directly under phase contrast microscopy. A clear difference in morphology was seen between the cells from the two bands (Figure 4.8). The cells from the upper band were regular rods of dimensions $1.0\ \mu\text{m} \times 2\text{-}4\ \mu\text{m}$. Refractive spore bodies were also observed in the upper band. The lower band cells were thinner, and had a wider range in length, $0.5\ \mu\text{m} \times 2\text{-}6\ \mu\text{m}$. The lower band also contained a number of the upper band cells, at a frequency of about 1:40. It was also noted that a high proportion of the upper band cells contaminating the lower band were sporulating.

Samples of each cell type were retained for protein analysis and transmission electron microscopy. Percoll was removed from these samples by diluting in the glycine buffer and centrifuging to produce a cell pellet.

Thin section transmission electromicrographs indicated that the upper band cells had a typical BC1 type morphology (Figure 4.9 a.), whereas those of the lower band resembled the second cell type seen previously in the ICC culture (Figure 4.9 b.).

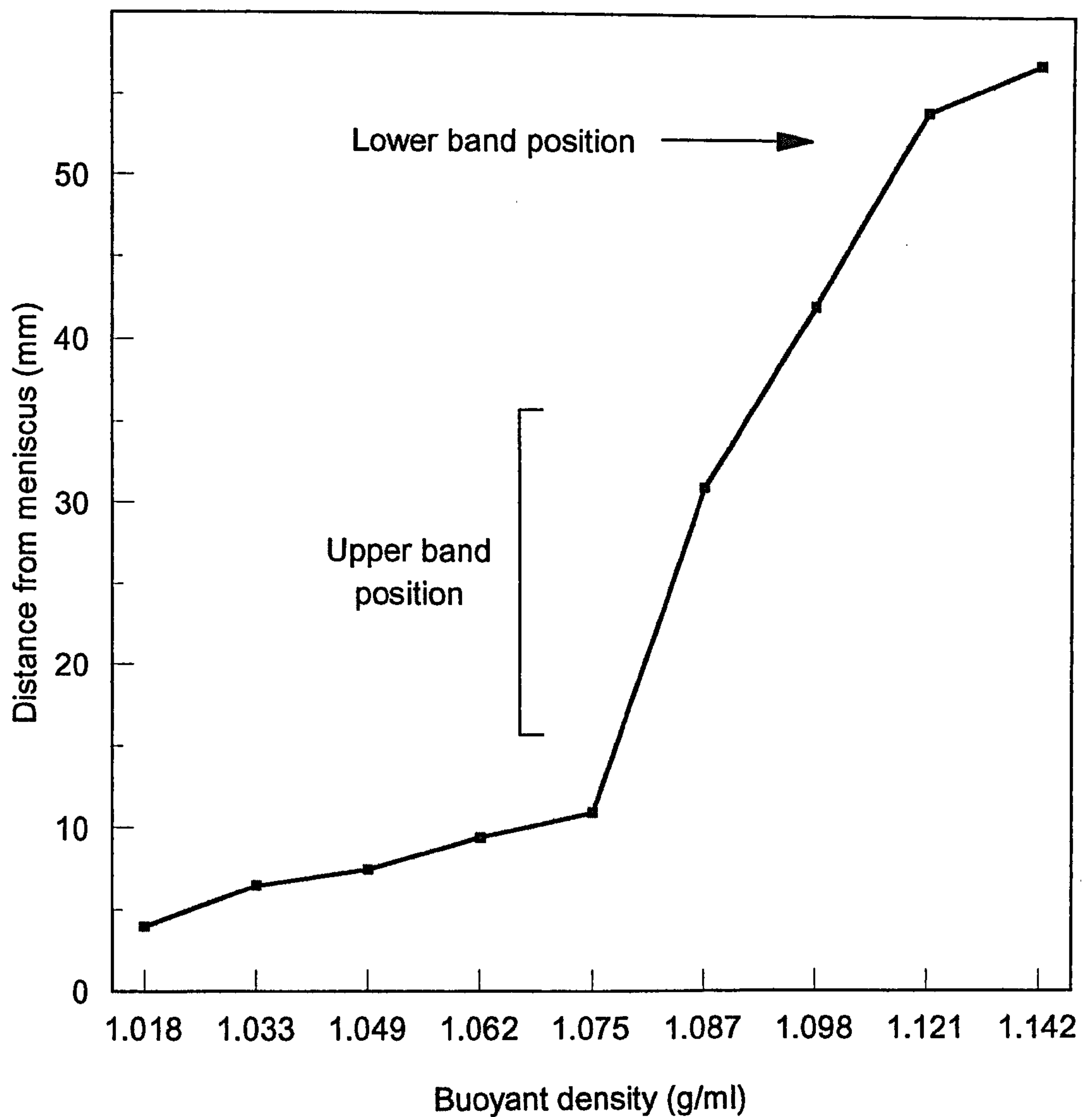
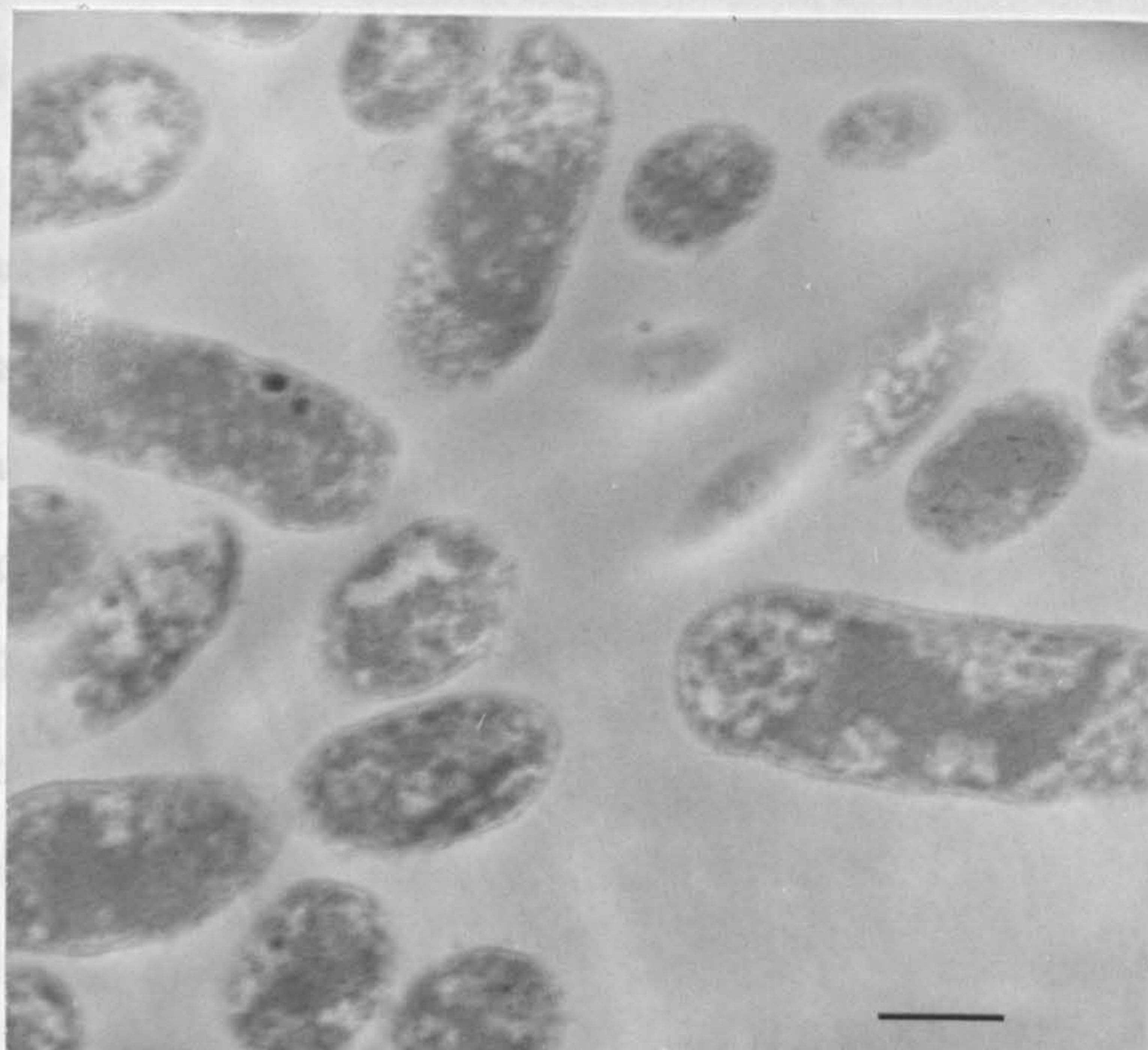


Figure 4.7: Location of bands of cells from culture ICC after Percoll gradient centrifugation of a cell suspension. The position of the two bands of cells are indicated on a 71% Percoll gradient in relation to the position of buoyant density marker beads.



Figure 4.8: Position of cell bands from autotrophically grown culture ICC on a 71% Percoll gradient. An upper diffuse band, and a smaller lower band are shown after centrifugation of autotrophically grown ICC cells on the Percoll gradient. Samples were extracted from each band using a hypodermic needle and viewed directly by phase contrast microscopy. The bar represents 5 μm .

a.



b.

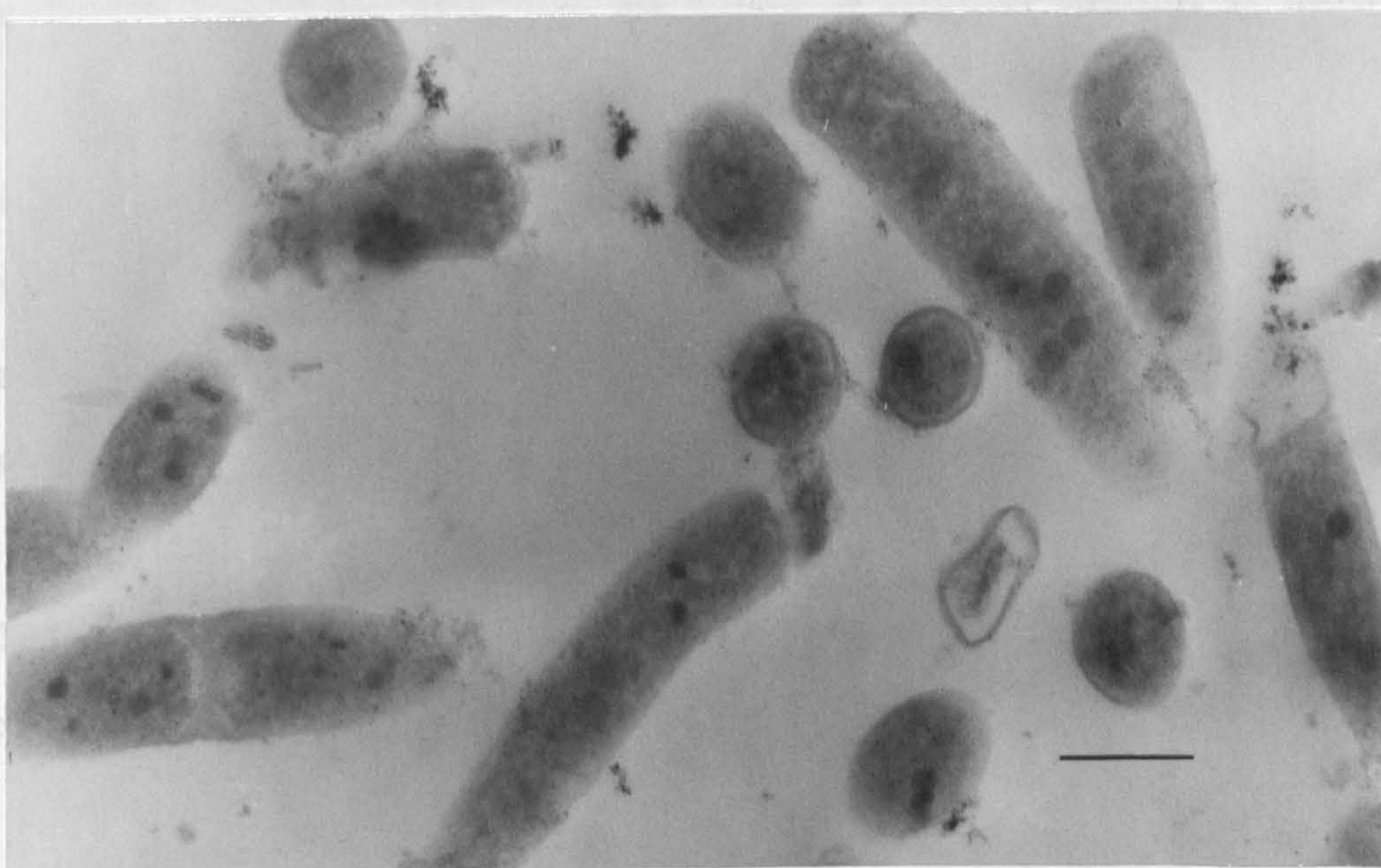


Figure 4.9: Thin section electron micrograph of cells from the upper (a), and lower (b) bands from a 71% Percoll gradient of autotrophically grown culture ICC. Thin sections were stained with uranyl acetate prior to transmission electron microscope analysis. Bar represents 0.5 μm .

Electrophoretic protein patterns also indicated a clear difference between the cells from the two bands (Figure 4.10). Samples were taken from two regions within the upper diffuse band, and from the lower band. The protein pattern remained identical down the length of the upper band, displaying high similarity to strain BC1. The protein profile of the lower band differed significantly from both strains BC1 and ALV.

Direct isolation from the gradients was attempted. Samples were plated onto ferrous iron plates with and without yeast extract. Colonies were formed from both bands, with autotrophic and chemolithoheterotrophic growth. The lower band colonies were very small (pin prick), and present in low numbers. A much larger number of colonies grew from the upper band, and these were also larger in size, about 1-2 mm in diameter. When colony samples were viewed directly by phase contrast microscopy no detectable difference was seen in cell morphologies. Electrophoretic protein patterns indicated high homology of isolates from both bands to strain BC1 (Figure 4.11), suggesting that the second cell type, predominant in the lower band, failed to grow on solid media. This was consistent with the failure of previous attempts to isolate the second cell type. Inoculation of liquid cultures directly from Percoll gradients also only resulted in BC1-type cultures. The harsh conditions encountered during Percoll gradient separations (i.e. high pH values, large number of washing steps and centrifugations) may have affected the viability of vegetative cells, with only spores of the BC1-type organism remaining viable.

Despite the simplicity of Percoll gradients, and the clear separation of the cell types that was achieved, the method ultimately failed to produce a viable pure culture of the second cell type.

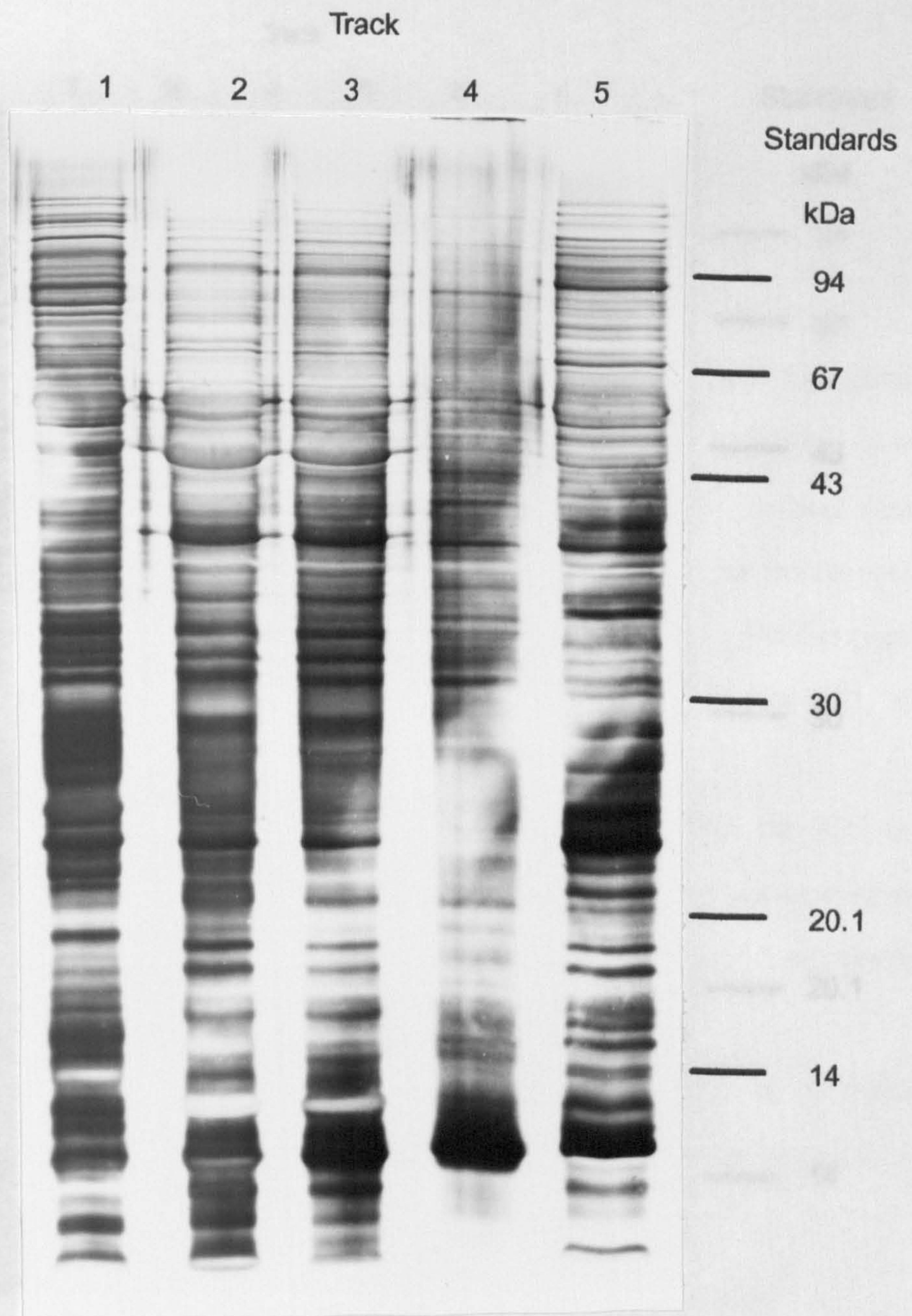


Figure 4.10: SDS-PAGE of samples extracted from a 71% Percoll gradient of autotrophically grown culture ICC. Samples were extracted from the Percoll gradient, using a hypodermic needle, washed to remove excess Percoll, and run on a 10% (w/v) denaturing polyacrylamide gel. The gel was then silver stained. Samples were extracted from two regions within the upper band of the Percoll gradient (tracks 2 and 3), and from the lower band (track 4). Strains ALV and BC1 are shown for comparative purposes (tracks 1 and 5 respectively).

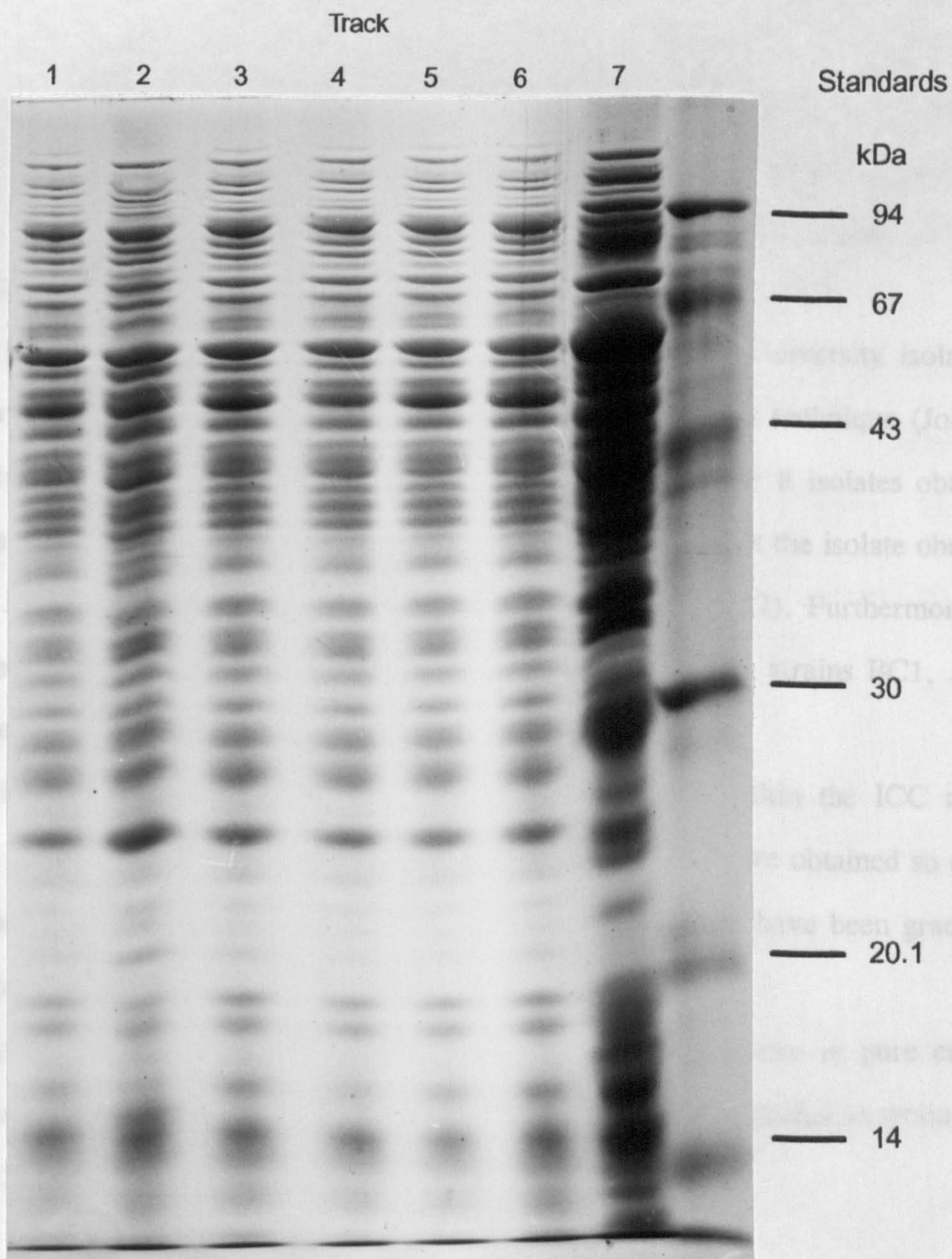


Figure 4.11: SDS-PAGE of isolates cultured from bands extracted from a 71% Percoll gradient of autotrophically grown culture ICC. Cell fractions were extracted from a Percoll gradient using a hypodermic needle, washed to remove excess Percoll, and then plated directly on to gelrite plates containing 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract. Single colonies were picked, cultured in liquid, and whole cell protein samples were run on a 10% (w/v) denaturing polyacrylamide gel, stained with Coomassie blue. Tracks 2, 3 and 4 were cultured from the lower band fraction of the Percoll gradient, tracks 5 and 6 from the upper band fraction of the gradient. Chemolithoheterotrophically grown strains BC1 and ALV are included for comparative purposes (tracks 1 and 6 respectively).

4.2.4: Isolation of novel strain ICP.

Whilst attempting separation of the two cell types within the ICC culture by Percoll gradients, the culture was routinely sub-cultured autotrophically on 50 mM ferrous iron supplemented with tetrathionate under air. After more than 35 sub-cultures, plating of the culture resulted in 8 isolates that grew autotrophically on ferrous iron, under air, at a faster rate than any other moderately thermophilic isolate in liquid culture, although to a lesser extent than seen with the ICC culture.

In parallel with this work, Dr. D. B. Johnson at Bangor University isolated a pure culture from the ICC mixed culture using an overlay plating technique (Johnson and McGinness, 1991). This culture grew at a similar rate to the 8 isolates obtained within this laboratory. Electrophoretic protein patterns indicated that the isolate obtained by D. B. Johnson was identical to the other 8 isolates (Figure 4.12). Furthermore, all these isolates had a protein pattern that differed substantially from strains BC1, ALV, and the mesophile *T. ferrooxidans*.

This new isolate appeared to be the second cell type within the ICC mixed culture. It is not clear why a number of isolates of this cell type were obtained so easily after so many failures. A good colony-forming strain may simply have been gradually selected for, during repeated sub-culturing.

Having obtained both cell types from the ICC mixed culture in pure culture further characterisation was carried out. The novel isolate was designated as strain ICP, and the BC1-type organism was designated strain ICH.

4.3: Characterization of novel isolates

A range of physiological and biochemical analyses were carried out to allow these preliminary characterisations to be compared with those previously (see Chapter 3).

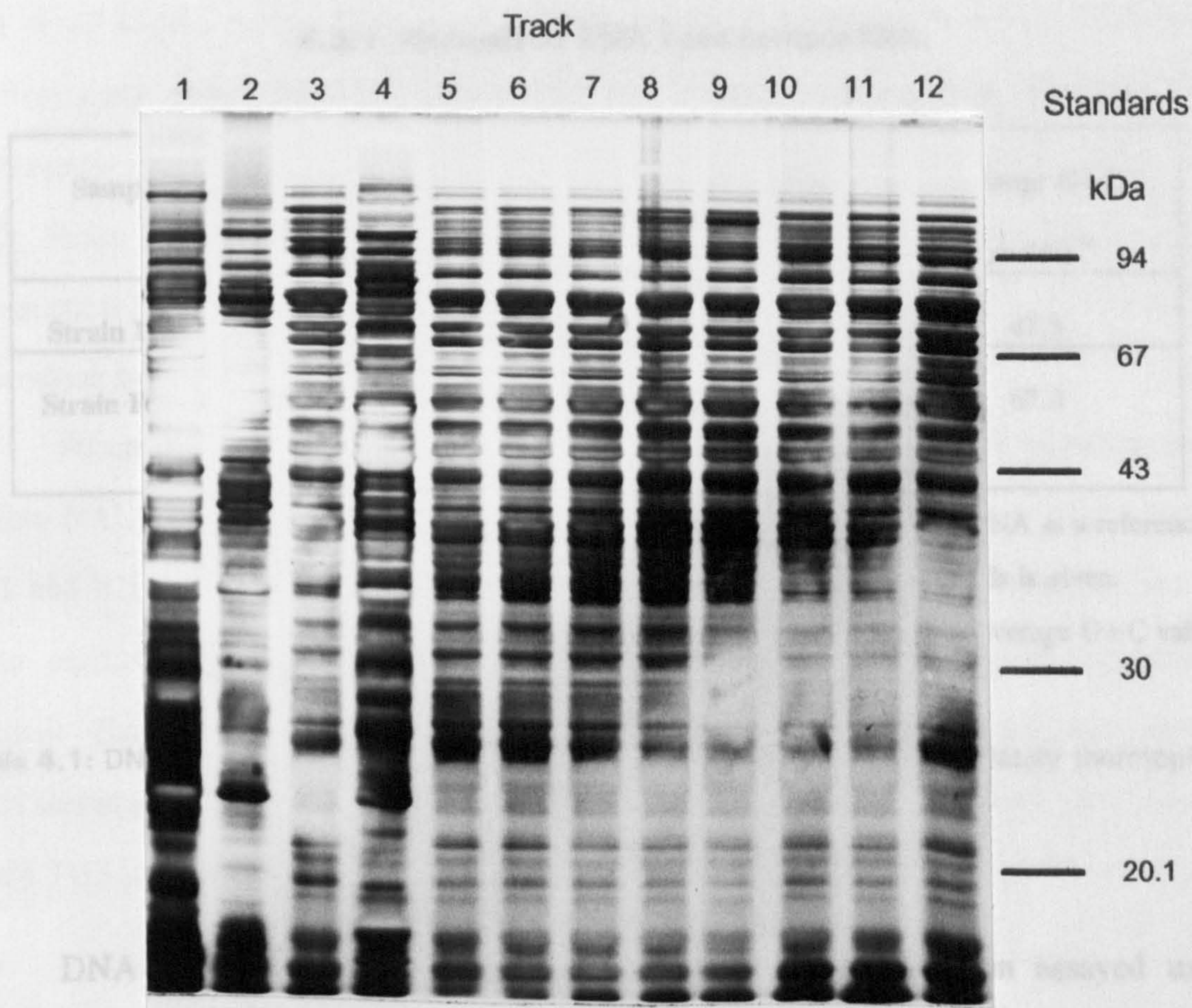


Figure 4.12: SDS-PAGE of novel isolates able to grow well autotrophically under air. Eight isolates were obtained from the ICC mixed culture via single colony isolation that were capable of good levels of growth autotrophically under air (tracks 5-12). In parallel to this work, Dr. D. B. Johnson at Bangor University, isolated a novel strain capable of comparable growth (track 3). Whole cell protein samples from these isolates, grown autotrophically, were run on a 10% (w/v) denaturing polyacrylamide gel, and silver stained. *Thiobacillus ferrooxidans* and autotrophically grown strains ALV and BC1 are included for comparative purposes (tracks 1,2 and 4 respectively).

4.3: Characterisation of novel isolates ICP and ICH.

A range of physiological and morphological studies of the novel isolates were carried out to allow their preliminary placement within the grouping scheme obtained previously (see Chapter 3).

4.3.1: Analysis of DNA base composition.

Sample	T _m (°C)	G+C (%) ^a	G+C (mol%) ^a	Average G+C (mol%) ^b
		<i>E. coli</i> standard	<i>M. luteus</i> standard	
Strain ICH	73.5	47.4	47.6	47.5
Strain ICP ^c	83.1	67.4	68.1	67.3
	82.7	66.6	67.3	

^a G+C value calculated using equation 3.3 and the stated standard DNA as a reference.

^b The average of the values obtained using the two reference standards is given.

^c Analysis of ICP DNA was carried out on duplicate samples. The average G+C value given is that for duplicate samples using both standards.

Table 4.1: DNA melting temperatures and base composition of novel moderately thermophilic iron-oxidizing bacteria.

DNA was extracted from the isolates, and base composition assayed using automated DNA melt testing, as carried out previously for a range of the moderate thermophiles (see section 3.4). The G+C values obtained for isolates ICP and ICH are given in table 4.1. Strain ICH had a G+C value of 47.5 mol% very similar to that of strain BC1 (48.2 mol%), confirming the close relationship between these two organisms. Isolate ICP had a much higher G+C value of 67.3 mol%. This value was almost identical to that obtained for strain TH3 (68.0 mol%), indicating a possible close relationship between these two isolates. This was an unexpected result, as strain TH3 had never been found anywhere apart from its original isolation site in New Mexico,

and it grew very poorly autotrophically, even with CO₂ enhancement (Norris and Barr, 1985).

4.3.2: Analysis of total cellular protein profiles.

Preliminary analysis of protein gels indicated a close relationship between strains ICH and BC1 (Fig. 4.4), and had also shown strain ICP to be unrelated to either strains BC1 or ALV (Fig 4.12). Analysis of protein gels using densitometry was carried out to confirm these initial findings. Strain ICH was compared to isolates from the three moderately thermophilic DNA:DNA hybridisation groups; strains BC1, NAL, and TH3. Strain ICP was compared only to strain TH3. Gels were analysed as previously (see section 3.3). The positions of the 20 most intense bands on the gel were again used to produce homology values for the isolates (Table 4.2).

Strain ICH was > 95% homologous to strain BC1, and < 70% homologous to strains NAL and TH3. This is confirmation of the close relationship between strains BC1 and ICH. The high homology between strains ICP and TH3 (>90%) confirms the close relationship between these two isolates indicated by DNA base composition analysis. Exact positioning of strain ICP within the grouping scheme was not possible from this single homology value, but it does suggest that ICP would group very close to strain TH3 in the third DNA:DNA hybridisation group (see Fig. 3.2).

Isolate	% Homology to:				
	Strain ICH	Strain ICP	Strain BC1	Strain NAL	Strain TH3
Strain ICH	100	-	95	64	39
Strain ICP	-	100	-	-	89
Strain BC1	97	-	100	-	-
Strain NAL	67	-	-	100	-
Strain TH3	42	90	-	-	100

Table 4.2: Homology values for novel moderately thermophilic iron-oxidizers based on the SDS-PAGE protein patterns.

4.3.3: Morphology of novel isolates.

As expected from DNA base composition and electrophoretic protein patterns, strain ICH showed morphological characteristics similar to those of strain BC1 (see section 3.5). During chemolithoheterotrophic growth strain ICH appeared as large rods $1.4\ \mu\text{m} \times 2.8\text{-}6.0\ \mu\text{m}$ singly, in pairs, or occasionally as short chains of cells (Fig. 4.13). Heterotrophically-grown strain ICH had the characteristic rounded morphology displayed by the *S. thermosulfidooxidans* hybridisation group, with cells of dimensions $1.4\text{-}1.8\ \mu\text{m} \times 2.0\text{-}3.5\ \mu\text{m}$ (Fig. 4.13). Cells were also similar in appearance to strain BC1 during autotrophic growth on iron, being more typical rods with smaller dimensions than seen during chemolithoheterotrophic growth, $0.9\ \mu\text{m} \times 2.2\text{-}2.8\ \mu\text{m}$ (Fig. 4.13).

Strain ICP cells were very small with all three growth conditions, with some similarities to strain TH3 (see Fig. 3.5). During chemolithoheterotrophic growth strain ICP had dimensions of $0.2\text{-}0.4\ \mu\text{m} \times 1.5\text{-}4\ \mu\text{m}$, but unlike strain TH3, which forms filaments, cells were single or in pairs (Fig. 4.13). During heterotrophic growth on yeast extract motile cells occurred singly or in pairs, $0.2\text{-}0.4\ \mu\text{m} \times 0.9\text{-}2.0\ \mu\text{m}$ in diameter (Fig. 4.13). Autotrophic growth on iron under enhanced CO_2 levels also

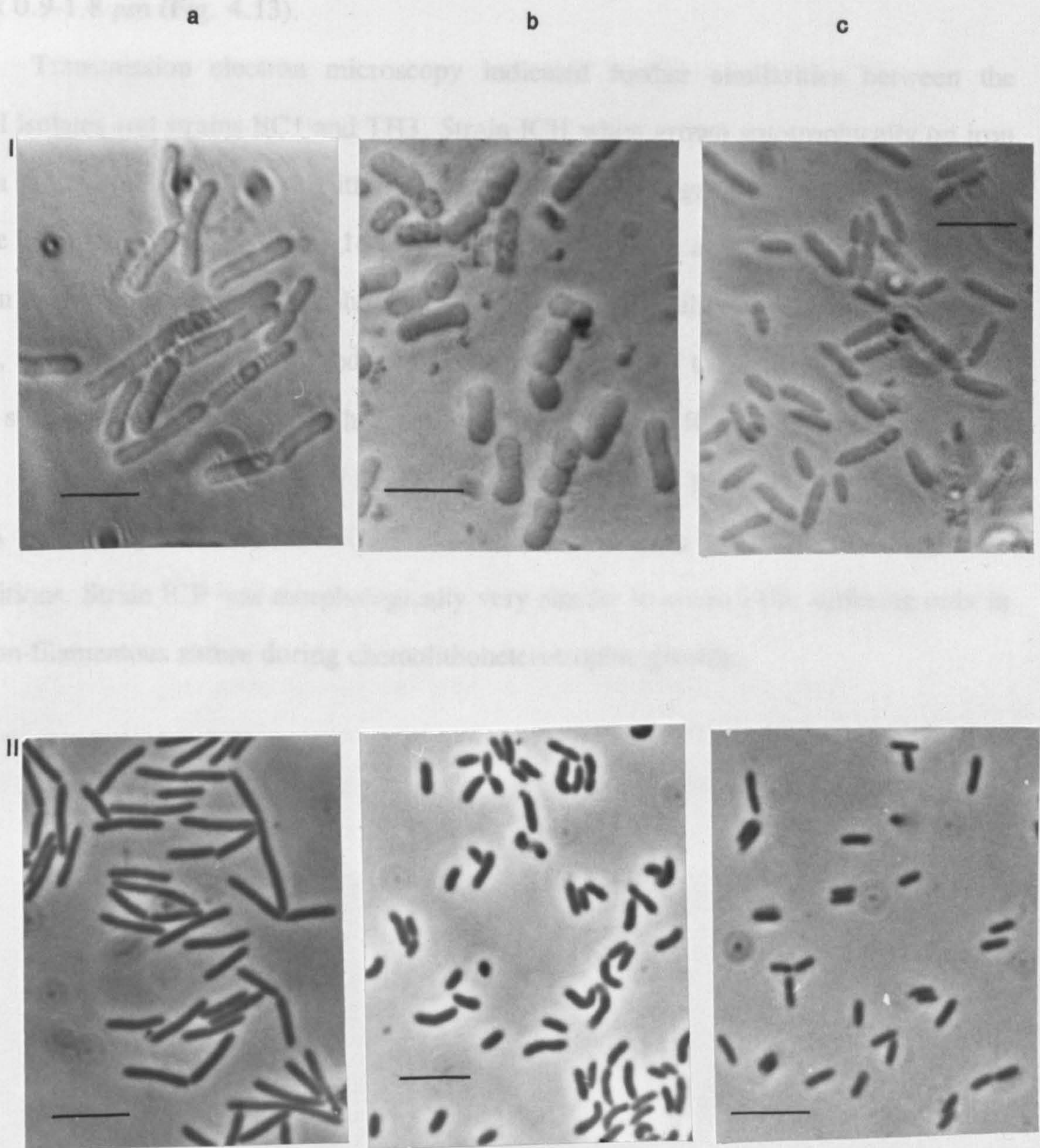


Figure 4.13: Phase contrast photomicrographs of novel moderately thermophilic bacteria strains ICH (I), and ICP (II). Bacteria were grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract (column a), heterotrophically on 0.02% (w/v) yeast extract (column b), and autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air (column c). The bar represents 5 μm.

resulted in a culture similar to strain TH3; a sparse population of very small rods 0.2 μm x 0.9-1.8 μm (Fig. 4.13).

Transmission electron microscopy indicated further similarities between the novel isolates and strains BC1 and TH3. Strain ICH when grown autotrophically on iron had a typical BC1 appearance with the accumulation of a negatively staining compound at the poles of the cell (Figure 4.14 a.). Spore formation was also noted with this strain. Strain ICP when grown autotrophically resulted in cells similar in appearance to strain TH3, with spherical inclusion bodies present at the poles of the cells (Figure 4.14 b.). Like strain TH3 spore formation has not been noted in strain ICP.

Morphological analysis of these two isolates supports the grouping suggested by DNA base composition. Strain ICH was almost identical to strain BC1 in all growth conditions. Strain ICP was morphologically very similar to strain TH3, differing only in its non-filamentous nature during chemolithoheterotrophic growth.

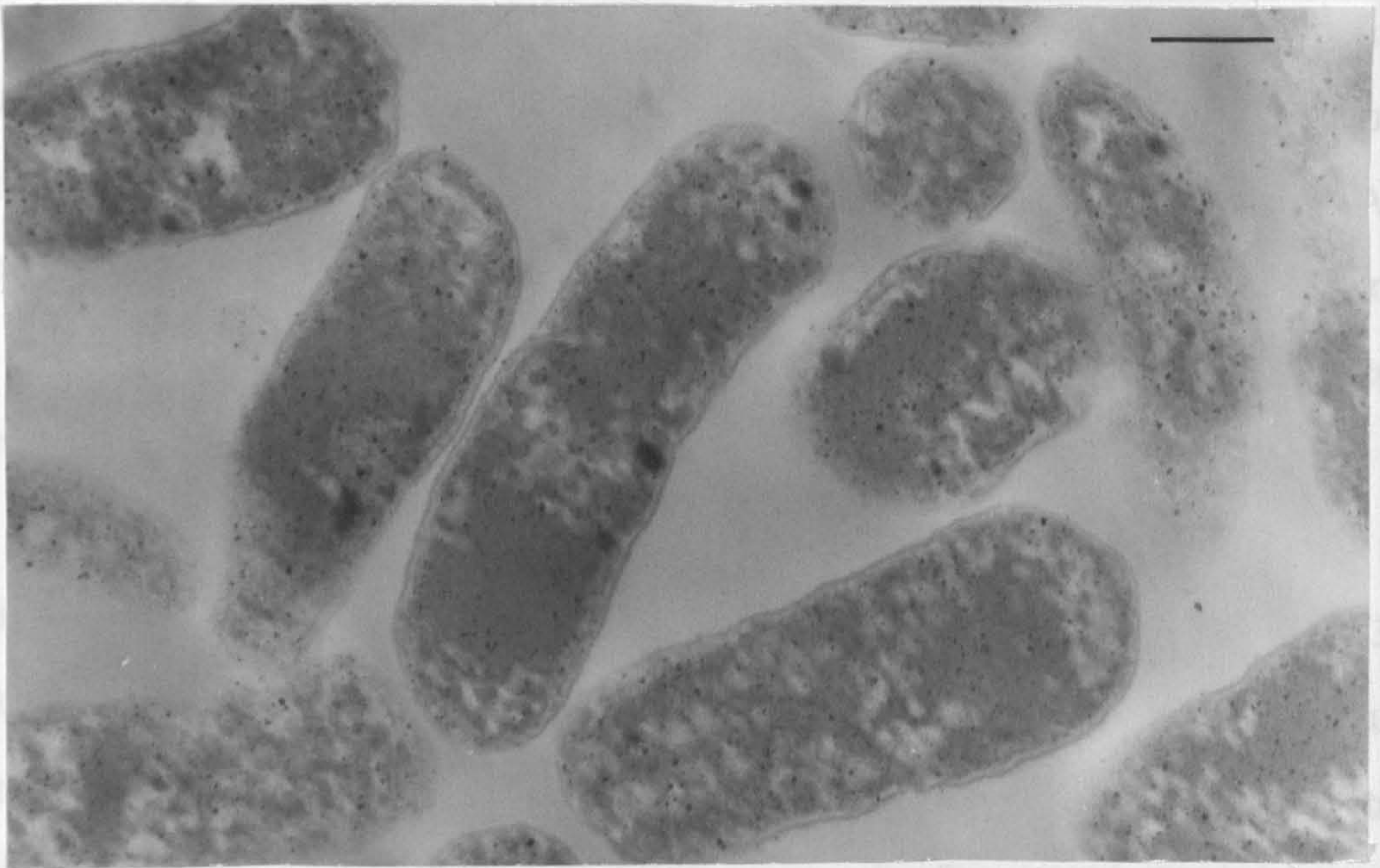


Figure 4.14 a: Thin section electron micrograph of strain ICH. Strain ICH was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% CO₂ (v/v) in air. Thin section analysis as for Fig. 4.3. Bar represents 0.5 μm.



Figure 4.14 b: Thin section electron micrograph of strain ICP. Strain ICP was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, under air. Thin section analysis as for Fig. 4.3. Bar represents 0.25 μm.

4.3.4: Physiology of novel isolates.

Heterotrophic growth of strain ICP on 0.02% (w/v) yeast extract resulted in a similar growth curve to strain TH3 (Figure 4.15), further confirmation of the close relationship between these two isolates. Strain ICH grew to a much better extent than strain BC1, but not to the level of growth achieved by any of the NAL DNA:DNA hybridisation group isolates. The growth of strain ICH, although better than strain BC1, was within the range observed for other isolates from the *S. thermosulfidooxidans* hybridisation group (Norris, unpublished data).

Antibiotic inhibition assays of the novel isolates were compared to the results obtained previously for other moderate thermophiles (see section 3.6.3). Heterotrophically-grown cultures were used, and so only zones of growth inhibition were measured (i.e. no iron oxidation zones). Only those antibiotics listed in table 3.4 that had previously shown clear differences among the three DNA:DNA hybridisation group organisms were used. Unfortunately, poor growth occurred with all isolates making measurement of zone sizes difficult. Figure 4.16 shows the inhibition zones for four of the antibiotics tested. Strain LM1 was not used in the previous assay, but is a typical *S. thermosulfidooxidans* group isolate. Strain ICH had a similar inhibition pattern to that shown by strain LM1; low sensitivity to 8 µg chloramphenicol, and high sensitivity to 20 µg ampicillin and 20 µg thiostrepton. This low sensitivity to chloramphenicol clearly distinguished strains ICH and LM1 from strain NAL in this experiment. However, a difference between the *S. thermosulfidooxidans* DNA:DNA hybridisation group, and the NAL DNA:DNA hybridisation group in their relative sensitivities to chloramphenicol was not noted in the previous studies (see section 3.6.3). Isolate ICP had a similar pattern of inhibition to that seen with strain TH3. Of the isolates tested, strains ICP and TH3 displayed the greatest resistance to 20 µg thiostrepton and the greatest sensitivity to 5 µg novobiocin.

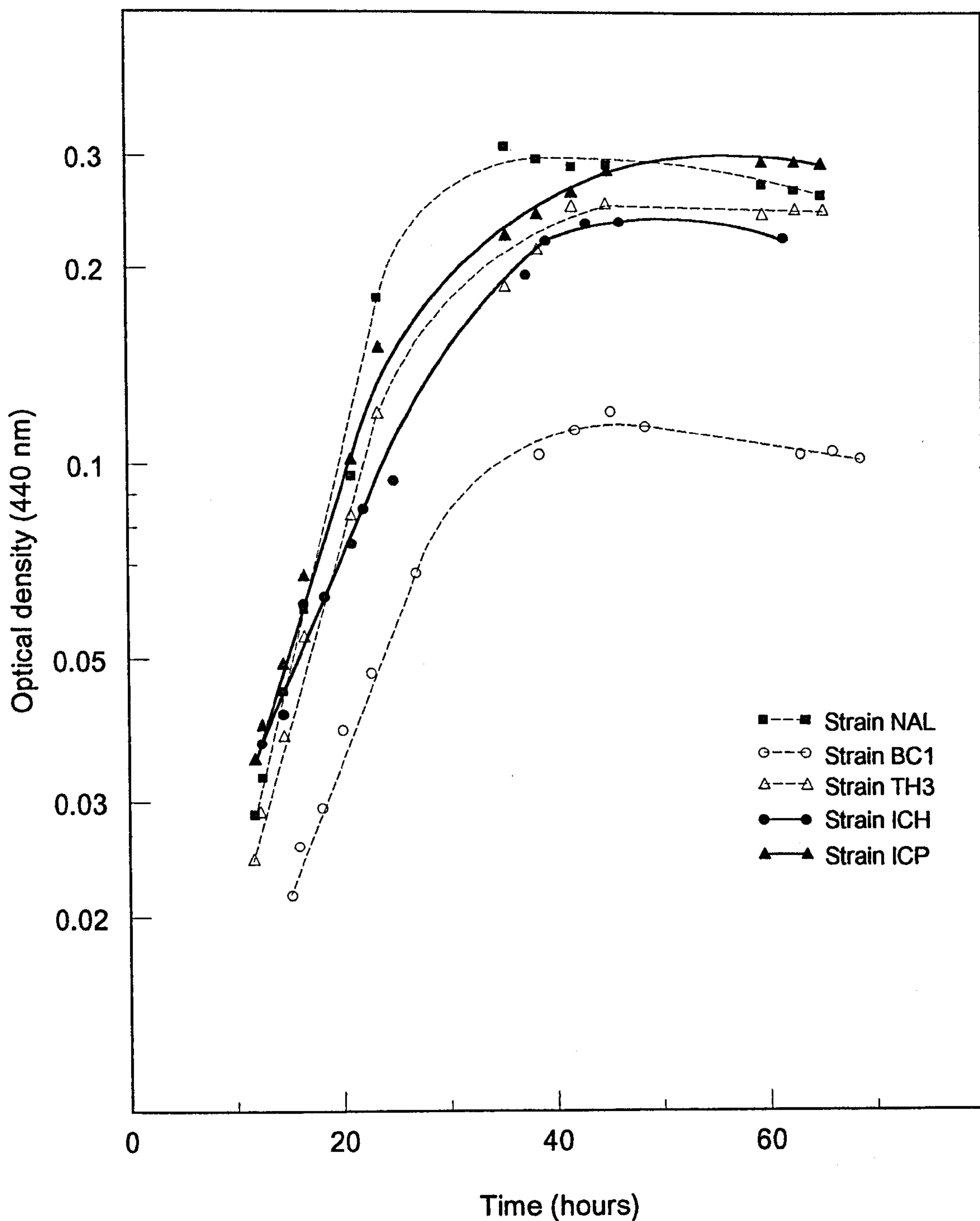


Figure 4.15: Heterotrophic growth of novel moderately thermophilic isolates in pH 2.0 medium containing 0.02% (w/v) yeast extract, at 48 °C. Growth of strains BC1, NAL, and TH3 are shown for comparison.

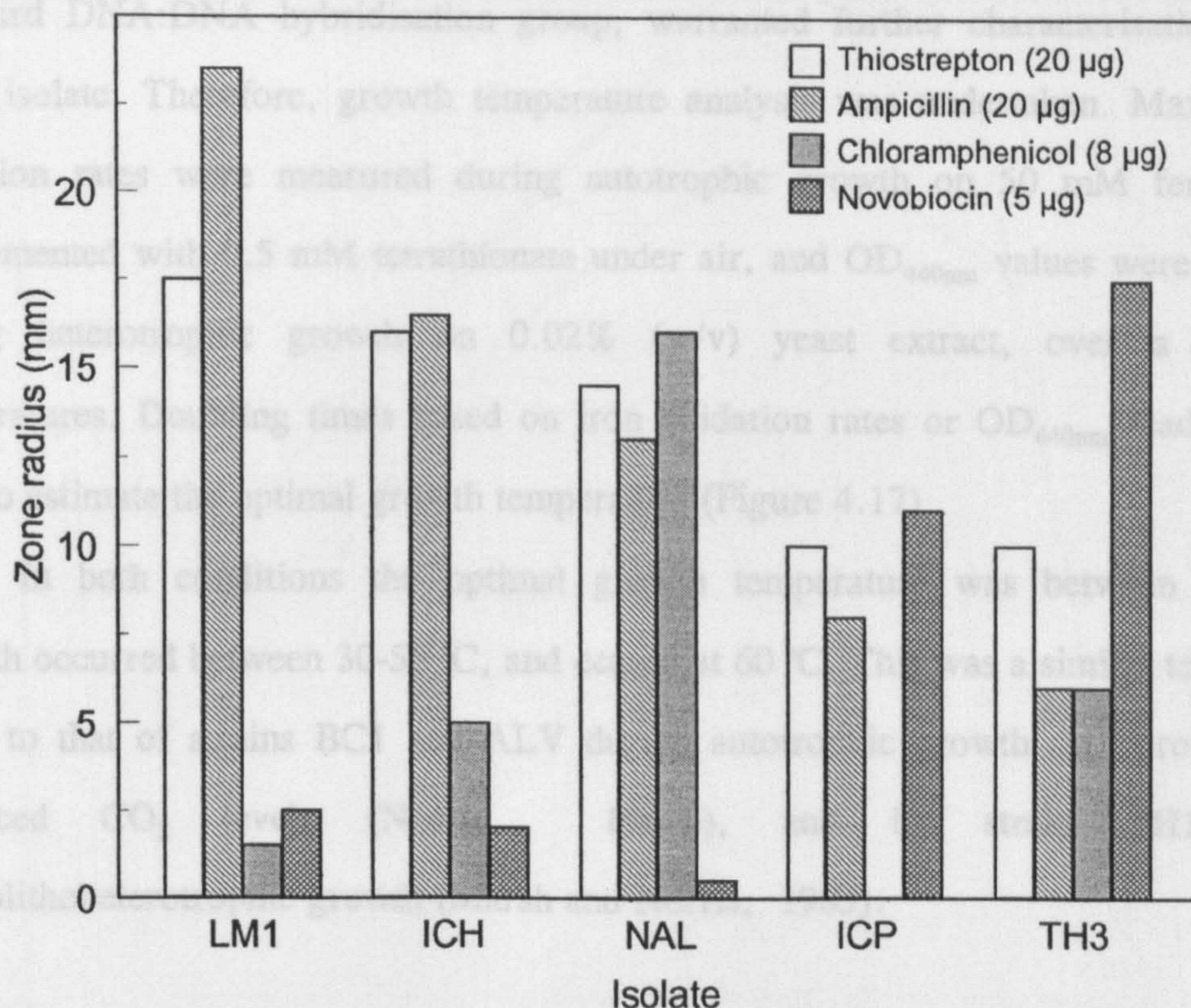


Figure 4.16: Zones of growth inhibition for novel moderately thermophilic iron-oxidizing isolates ICH and ICP compared to representatives from the three different DNA:DNA hybridisation groups in antibiotic diffusion assays.

Pour plates contained 1% (v/v) inoculum of exponentially growing cells, 0.02% (w/v) yeast extract and salts at pH 2.2. Plates were incubated at 48°C for 6 days. The graph illustrates the sizes of growth inhibition zones produced by antibiotic assay discs (Mast Laboratories, Liverpool). The radius of the zone was measured from the edge of the disc to the periphery of the zone. Strain LM1 is a *S. thermosulfidooxidans* group isolate, strain NAL is an isolate from the second DNA:DNA hybridisation group, and strain TH3 from the third group. Antibiotics and the amount used are indicated. No growth could be seen on the strain ICP chloramphenicol plate, and so this bar has been omitted.

The fact that strain ICP was only the second isolate discovered that belonged to the third DNA:DNA hybridisation group, warranted further characterisation of this novel isolate. Therefore, growth temperature analysis was undertaken. Maximal iron oxidation rates were measured during autotrophic growth on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under air, and OD_{440nm} values were measured during heterotrophic growth on 0.02% (w/v) yeast extract, over a range of temperatures. Doubling times based on iron oxidation rates or OD_{440nm} readings were used to estimate the optimal growth temperature (Figure 4.17).

In both conditions the optimal growth temperature was between 43-50 °C. Growth occurred between 30-55 °C, and ceased at 60 °C. This was a similar temperature range to that of strains BC1 and ALV during autotrophic growth on ferrous iron at enhanced CO₂ levels (Norris, 1989b), and by strain TH1 during chemolithoheterotrophic growth (Marsh and Norris, 1983).

Characterisation of the isolates obtained from the ICC mixed culture revealed strain ICH to be a typical *S. thermosulfidooxidans* group organism, whilst strain ICP was closely related to strain TH3 and as such is only the second isolate to be placed in the third DNA:DNA hybridisation group.

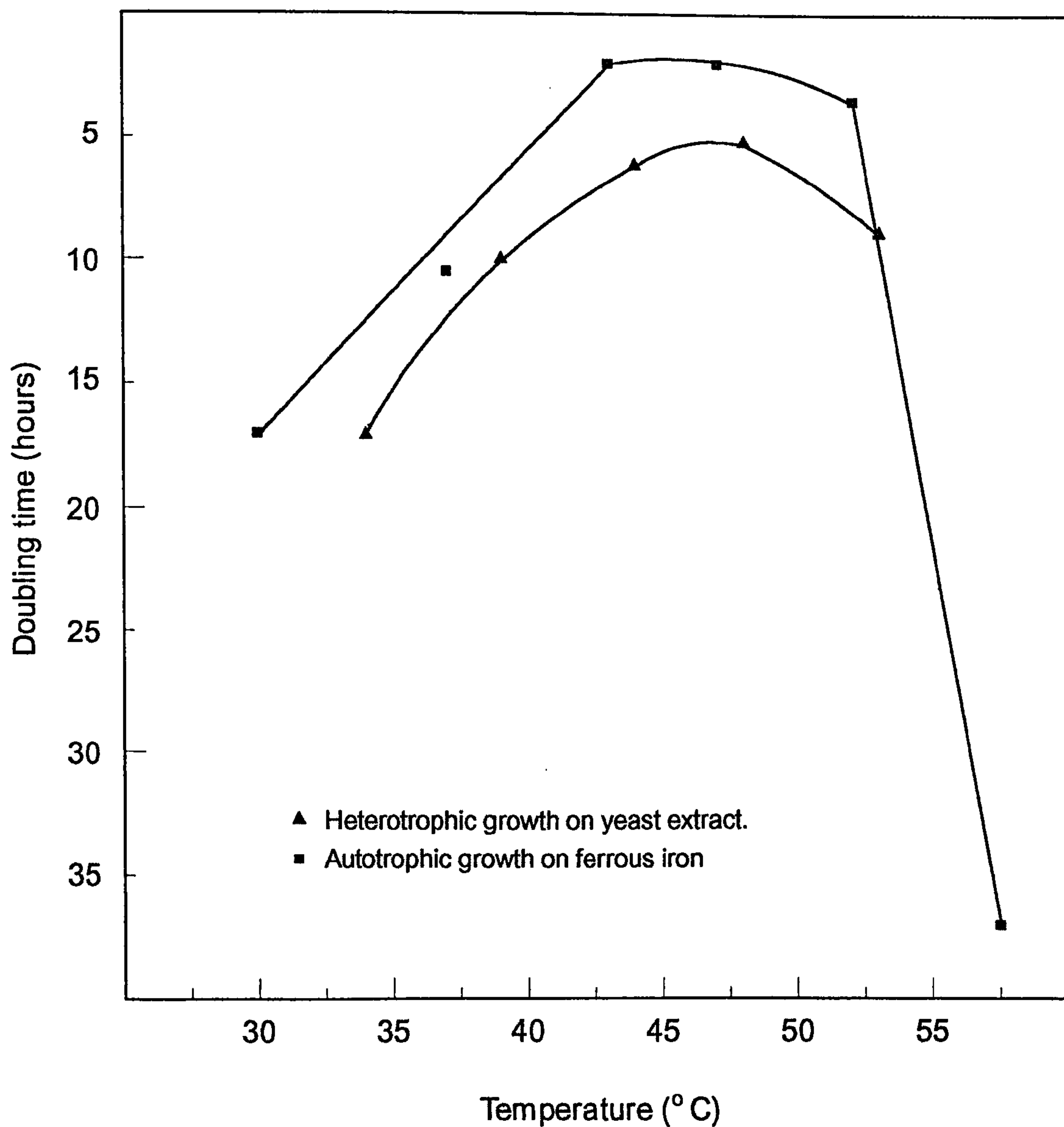


Figure 4.17: The effect of temperature on the growth of novel isolate ICP during heterotrophic growth on 0.02% (w/v) yeast extract at pH 2.0, and autotrophic growth on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at pH 1.7, under air. Doubling times refer to optical density (440 nm), and iron solubilisation respectively.

4.4: Study of the interactions within the ICC mixed culture.

Having established that the ICC mixed culture consisted of at least two distinct cell populations, the relative growth of each organism in mixed culture under air was investigated.

4.4.1: Percoll gradient centrifugation with novel isolates.

Percoll gradients prior to the isolation of novel isolate ICP indicated the presence of two cell populations within the ICC culture. The first of these isolates was identified as a BC1-type organism, but the second was not identified at the time. Before further work on the mixed culture, Percoll gradients of the pure ICP and ICH cultures were carried out, to confirm that strain ICP was the second cell type seen previously on ICC Percoll gradients. Autotrophically-grown cultures of strains ICP and ICH were harvested, washed, and placed on separate 71% Percoll gradients. Gradients were centrifuged as before (see section 2.10), and compared to a fresh gradient of the ICC mixed culture. Figure 4.18 shows the results of these three gradients. The ICP culture produced a single band about 50 mm from the top of the gradient (Fig. 4.18 a.), corresponding to the position of the lower band on the ICC gradient (Fig. 4.18 c.). The ICH culture produced a diffuse band of cells immediately below the cell debris layer at the top of the gradient (Fig. 4.18 b.), corresponding to the upper band on the ICC gradient (Fig. 4.18 c.).

The cell bands were extracted from the ICC mixed culture gradient, and electrophoretic patterns compared to strains ICP and ICH by SDS-PAGE (Figure 4.19). The lower band cells showed high similarity to strain ICP, whereas the upper band cells showed greater similarity to strain ICH, although in both cases some differences were visible. The differences in patterns between cells extracted from the gradient, and the pure cultures were probably a result of the cross-contaminated nature of bands extracted from Percoll gradients.

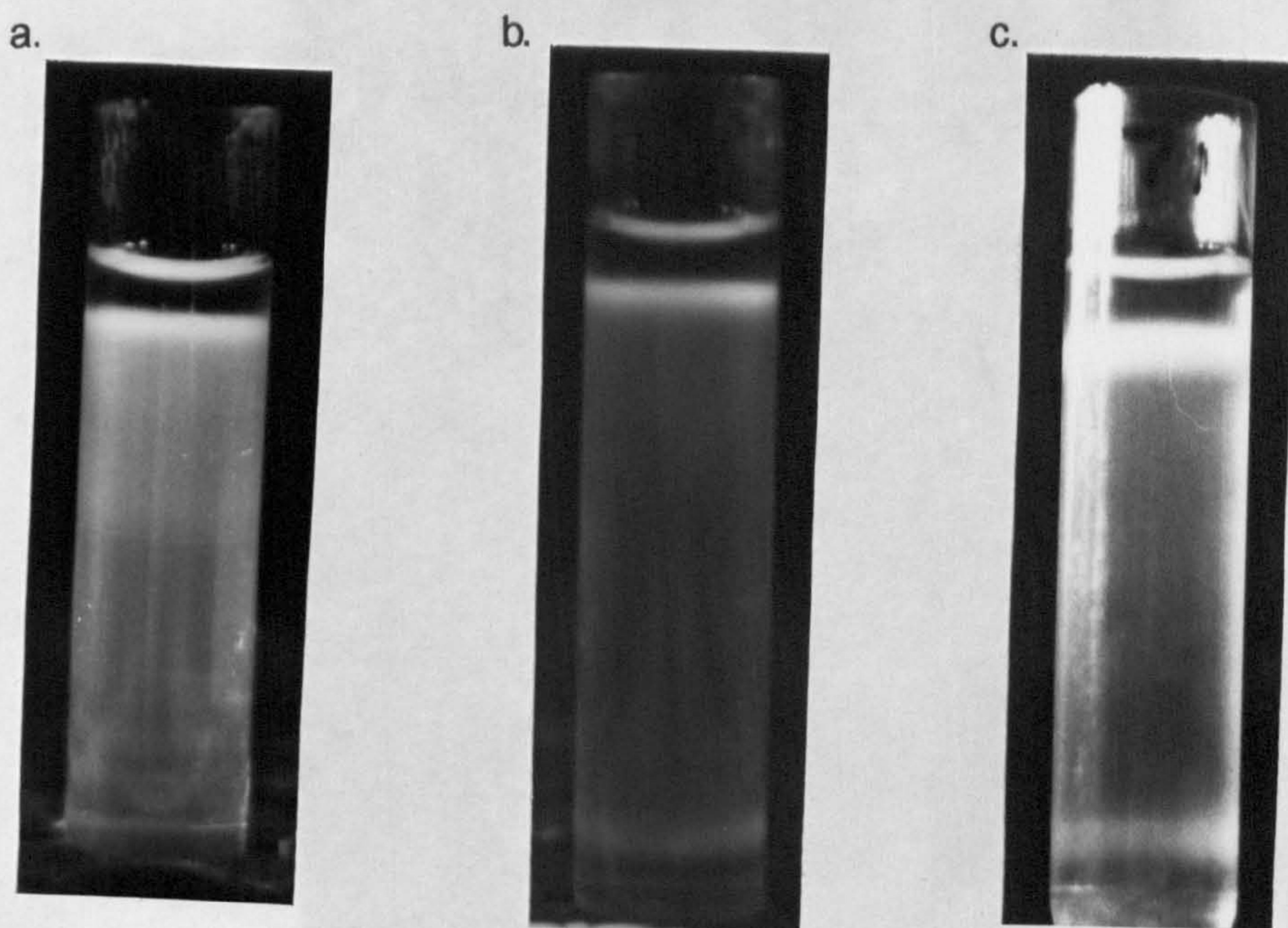


Figure 4.18: Comparison of banding patterns of strains ICH (a), ICP (b) and the mixed culture ICC (c) on a 71% Percoll gradient. Cultures were grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, under air, harvested by centrifugation, and loaded on to separate 71% Percoll gradients.

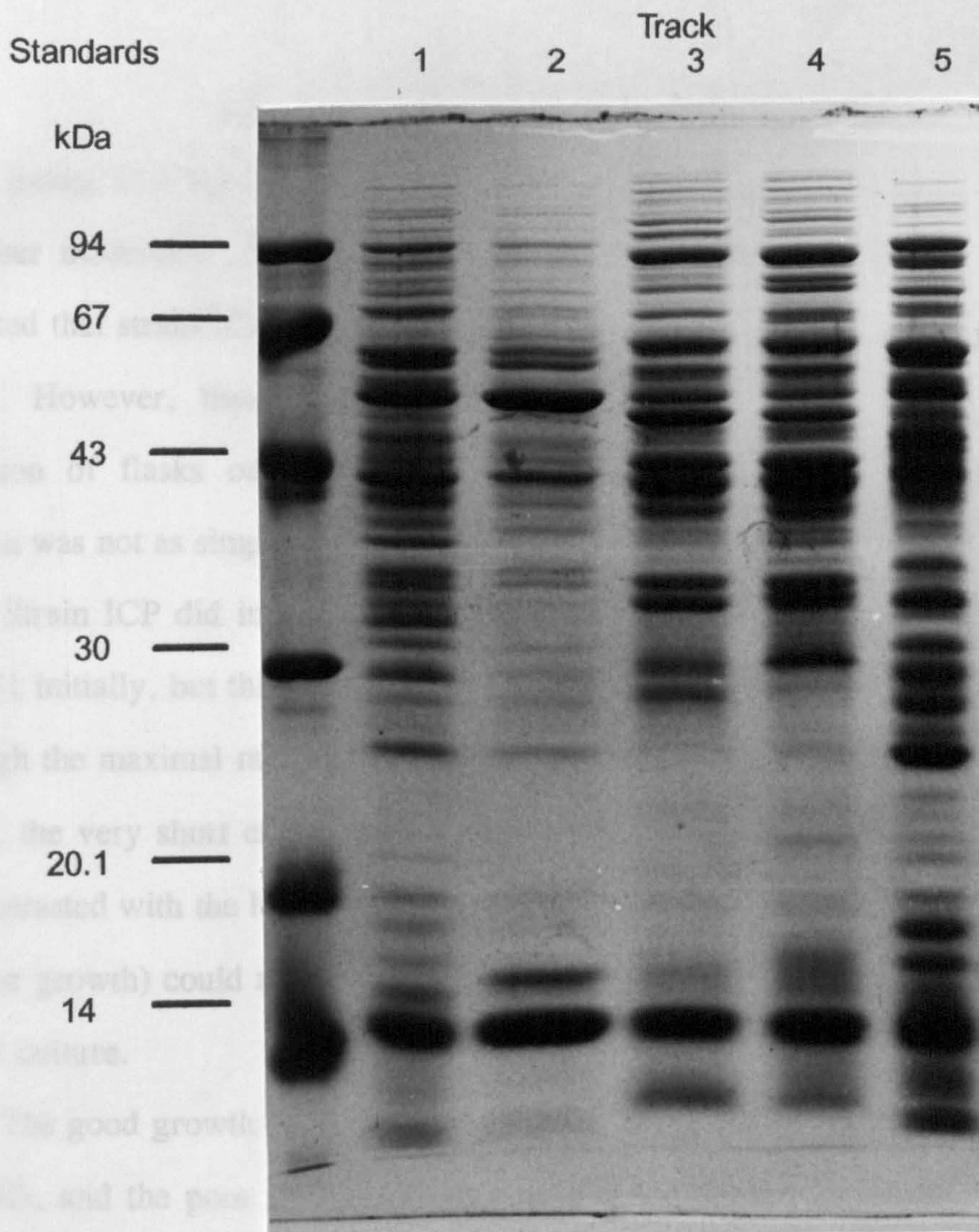


Figure 4.19: SDS-PAGE of novel isolates ICH (track 5) and ICP (track 4) compared to the upper (track 2), and lower (track 3) cell bands extracted from a 71% Percoll gradient of culture ICC. Strains ICH, ICP and culture ICC were grown autotrophically on ferrous iron. Cell bands from the Percoll gradient were extracted with a hypodermic needle and washed to remove excess Percoll. Whole cell protein samples were prepared, run on a 10% (w/v) denaturing polyacrylamide gel, and Coomassie stained. Autotrophically grown strain BC1 (track 1) is shown for comparative purposes.

These gradients showed the pattern expected assuming strain ICP was the second cell type seen previously during Percoll gradient experiments. The ICC mixed culture clearly consists of only two organisms, strains ICP and ICH, with the autotrophic growth in air being a consequence of one or both of these isolates.

4.4.2: Autotrophic growth of the novel isolates.

Isolate ICP was capable of iron oxidation to a level greater than that achieved by any other moderately thermophilic isolate during autotrophic growth under air. This suggested that strain ICP was responsible for the good autotrophic growth of the mixed culture. However, these observations of growth were based on qualitative visual inspection of flasks only. When quantitative measurements were carried out, the situation was not as simple as initially believed.

Strain ICP did indeed oxidize iron at a much faster rate than both strains ICH and BC1 initially, but this high rate of oxidation was for a short duration (Figure 4.20). Although the maximal rate of oxidation was equivalent to the rates seen with the mixed culture, the very short exponential phase of growth, and the rapid decline in oxidation rate contrasted with the levels achieved by culture ICC. The growth of strain ICH under air (poor growth) could not on its own have contributed to the superior performance of the ICC culture.

The good growth of the ICC mixed culture (which consisted only of strains ICP and ICH), and the poor growth of strains ICP and ICH in pure culture indicated that autotrophic growth under air was a mixed culture phenomenon. To test this hypothesis, co-culture experiments were carried out. Cultures were grown autotrophically under air, either as pure cultures, or in co-culture with a second moderately thermophilic iron-oxidizing isolate.

A co-culture consisting of strain ICP and either strain BC1 or strain NAL produced superior iron oxidation to that in any of the pure cultures (Figure 4.21). The oxidation reached 80-100% compared to only 40-50% by ICP alone. Strains BC1 and NAL in pure culture and co-culture with one another produced poor iron oxidation rates

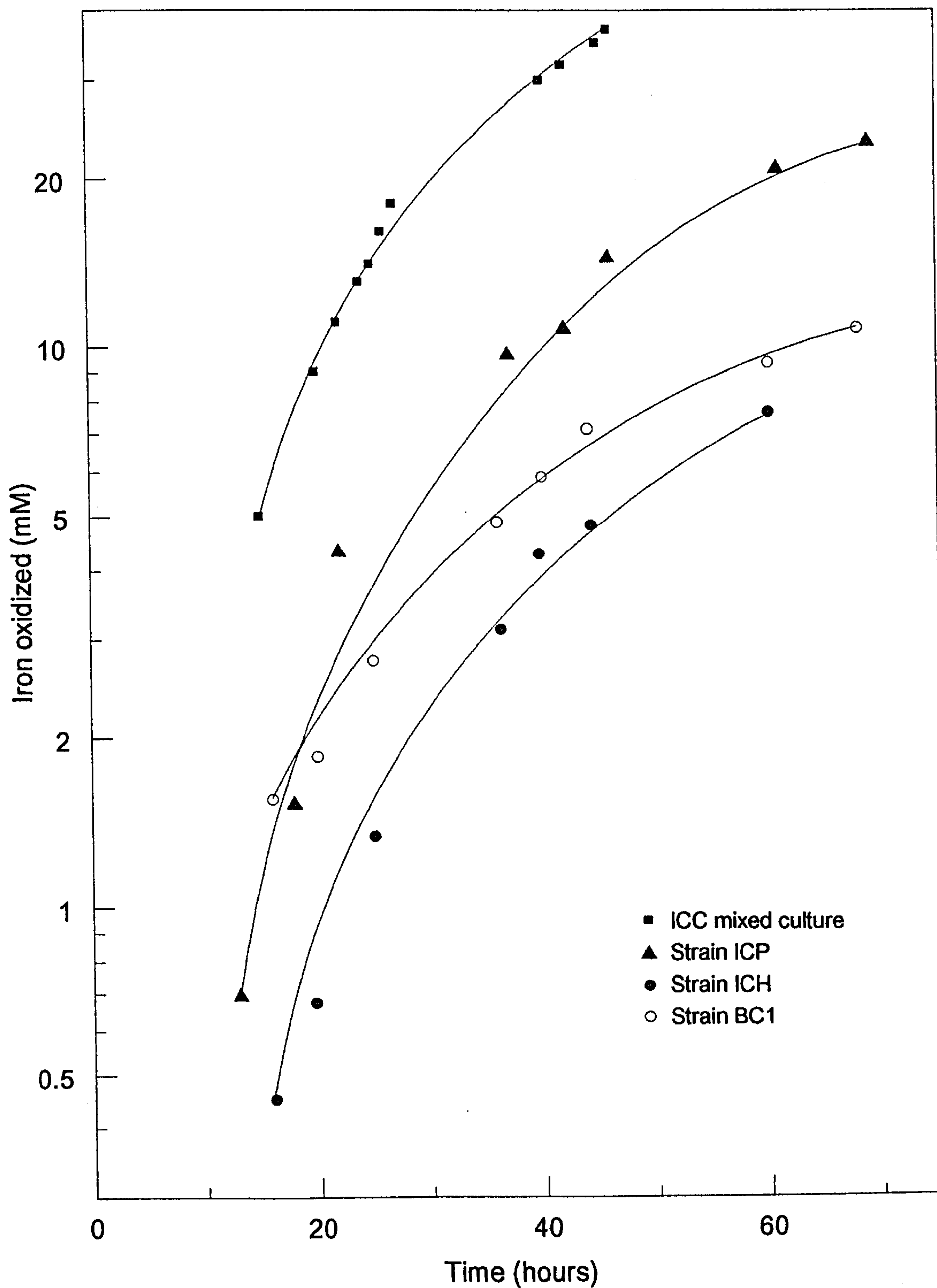


Figure 4.20: Iron oxidation during autotrophic growth of four moderately thermophilic iron-oxidizing cultures in pH 1.7 medium containing 50 mM ferrous iron supplemented with 0.05 mM tetrathionate, under air, at 48 °C.

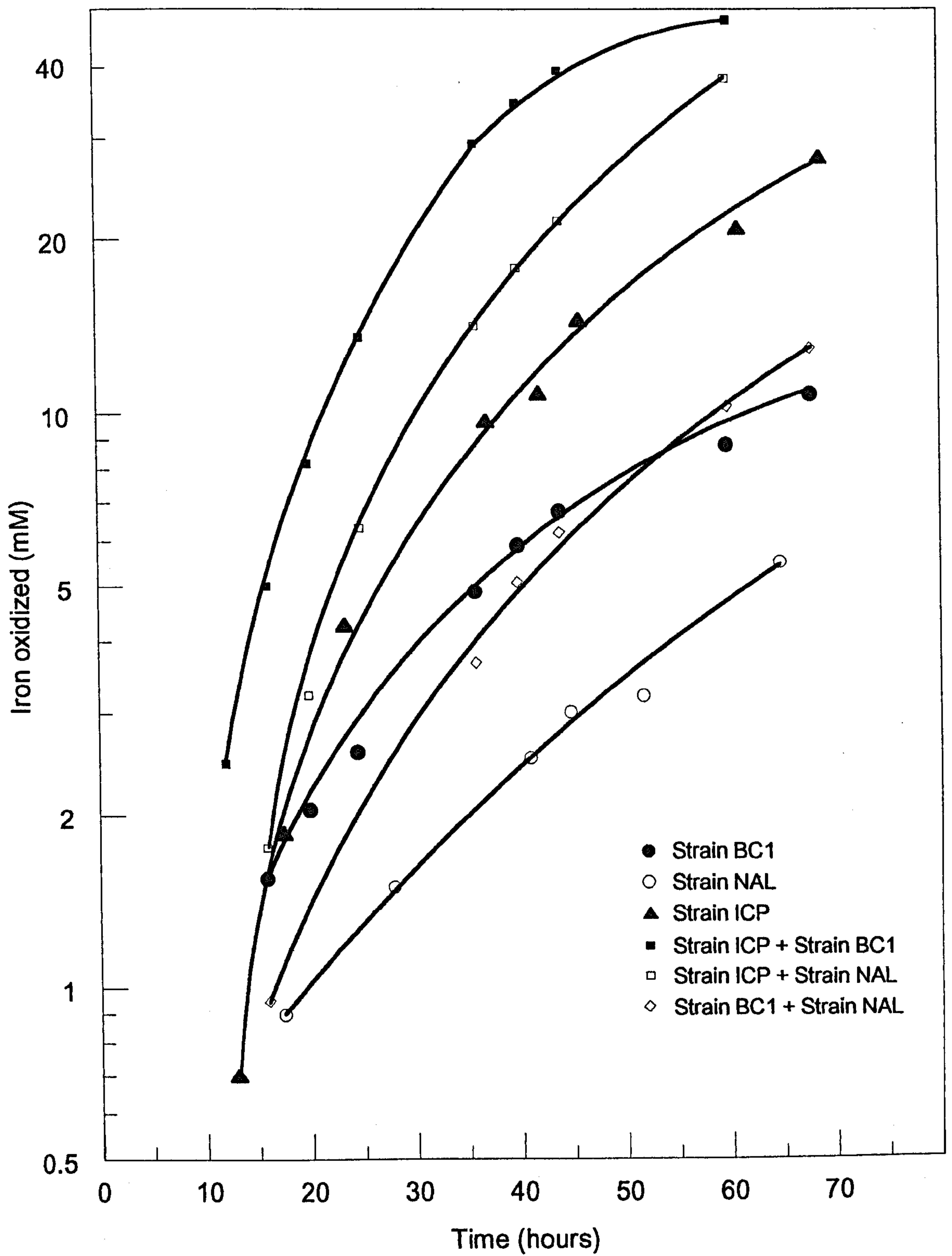


Figure 4.21: Iron oxidation during autotrophic growth of three moderately thermophilic iron-oxidizing bacteria in pure and mixed cultures, under air. All cultures were grown at 48°C on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, in pH 1.7 medium.

and yields. Similar results were obtained for co-cultures consisting of strain ICH instead of either strains BC1 or NAL, i.e. good growth in combination with strain ICP, but poor growth in any other combination.

Strain ICP was essential to achieve high rates of iron oxidation in these co-cultures, but the second organism within the co-culture could be a member of either the *S. thermosulfidooxidans* DNA:DNA hybridisation group or the NAL DNA:DNA hybridisation group. However, it was possible to replace strain ICP with strain TH3 without affecting the oxidation levels.

A co-culture consisting of strain ICP and an acidophilic obligate heterotroph, *Bacillus* K2, failed to produce oxidation levels above those achieved by strain ICP alone (data not shown), suggesting that two iron-oxidizing bacteria were necessary for efficient iron oxidation.

Similar co-culture experiments were attempted using pyrite mineral as substrate, but a co-culture of strains ICP and BC1 or ICH did not result in any significantly higher mineral leaching rates autotrophically under air than the pure cultures, whereas the ICC mixed culture did. This failure of co-cultures to oxidize mineral complicates the situation (and requires further investigation), but the interaction between strain ICP and a second iron-oxidizer is still believed to be the basis of this consortium's ability to grow autotrophically under air.

4.4.3: Affinity of isolate ICP for iron.

The essential role strain ICP plays within the ICC mixed culture is believed to be associated with efficient CO₂ utilisation (see chapter 5). However, the rapid decline in oxidation rate after only about 10-15% of the ferrous iron was oxidized clearly suggests that strain ICP becomes inhibited. Strain ALV is known to grow poorly autotrophically on minerals (Norris and Barr, 1985). This poor growth was believed to be associated with a higher affinity for the end product of iron oxidation, ferric iron, than the substrate, ferrous iron (Wood *et al.*, 1987). Therefore, growth of strain ICP in the presence of ferric iron was investigated to see if a similar phenomenon was involved.

In the earlier work, growth of strain ALV was reduced by 20 mM ferric iron and completely inhibited by 50 mM, whereas strain BC1 remained relatively unaffected even by the addition of 50 mM ferric iron (Wood *et al.*, 1987). Strain ICP was affected by ferric iron in a similar manner to strain ALV (Figure 4.22). 20 mM ferric iron, twice the ferrous iron concentration, reduced oxidation by strain ICP, and 50 mM ferric iron stopped oxidation almost completely. Strain BC1 was relatively little affected by the ferric iron (Fig. 4.22).

Oxygen electrode studies have been used to determine the apparent Michaelis constant (K_m) for iron oxidation by cell suspensions of mesophilic iron-oxidizing bacteria (Kelly and Jones, 1978). An oxygen electrode was used to determine the oxygen uptake by cell suspensions of autotrophically- and chemolithoheterotrophically-grown strain ICP, and *S. thermosulfidooxidans* cells at different ferrous iron concentrations, in the presence or absence of ferric iron. Differences in iron oxidation kinetics were then related to the growth curves.

Chemolithoheterotrophically-grown strain ICP cells had an apparent K_m of 0.24 mM ferrous iron calculated from double reciprocal plots (Figure 4.23). The addition of ferric iron (10 mM) resulted in competitive inhibition, with a calculated inhibitor constant (K_i) of 1.44 mM ferric iron. All inhibitor constants were calculated using equation 4.1:

$$K_m' = K_m (1 + i/K_i) \quad (4.1)$$

Where K_m' = Michaelis constant during inhibition.

K_m = Michaelis constant in the absence of inhibitor.

i = inhibitor concentration.

K_i = inhibitor constant.

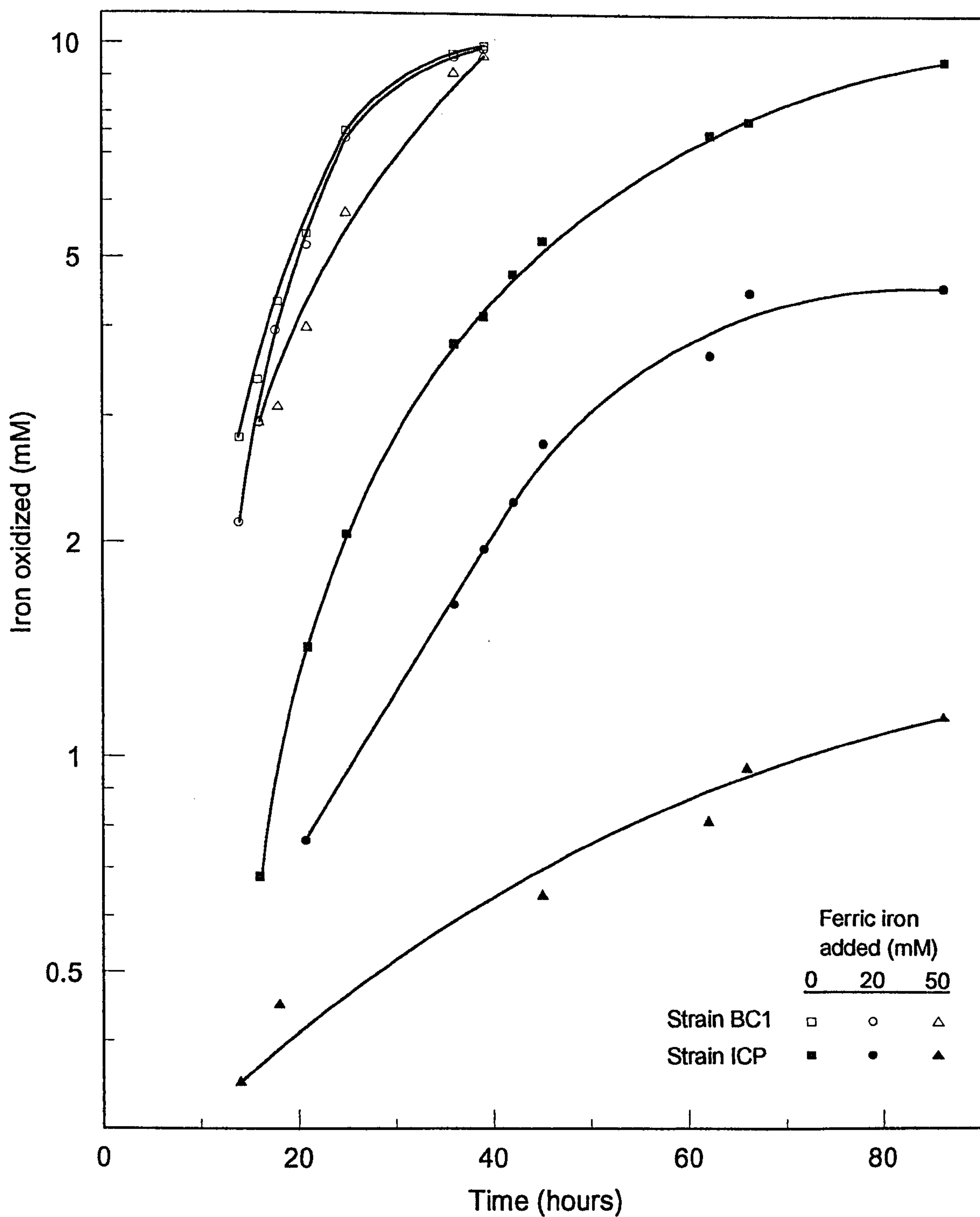


Figure 4.22: Iron oxidation during autotrophic growth of two moderately thermophilic iron-oxidizing bacteria on 10 mM ferrous iron, after the addition of 0, 20 or 50 mM ferric iron. All cultures were grown in pH 1.7 medium supplemented with 0.5 mM tetrathionate at 48 °C, under 1% (v/v) CO₂ in air.

$$V_{\max} = 3.19 \text{ } \mu\text{moles O}_2/\text{min/mg protein}$$

$$K_m = 0.24 \text{ mM}$$

$$K_i = 1.44 \text{ mM}$$

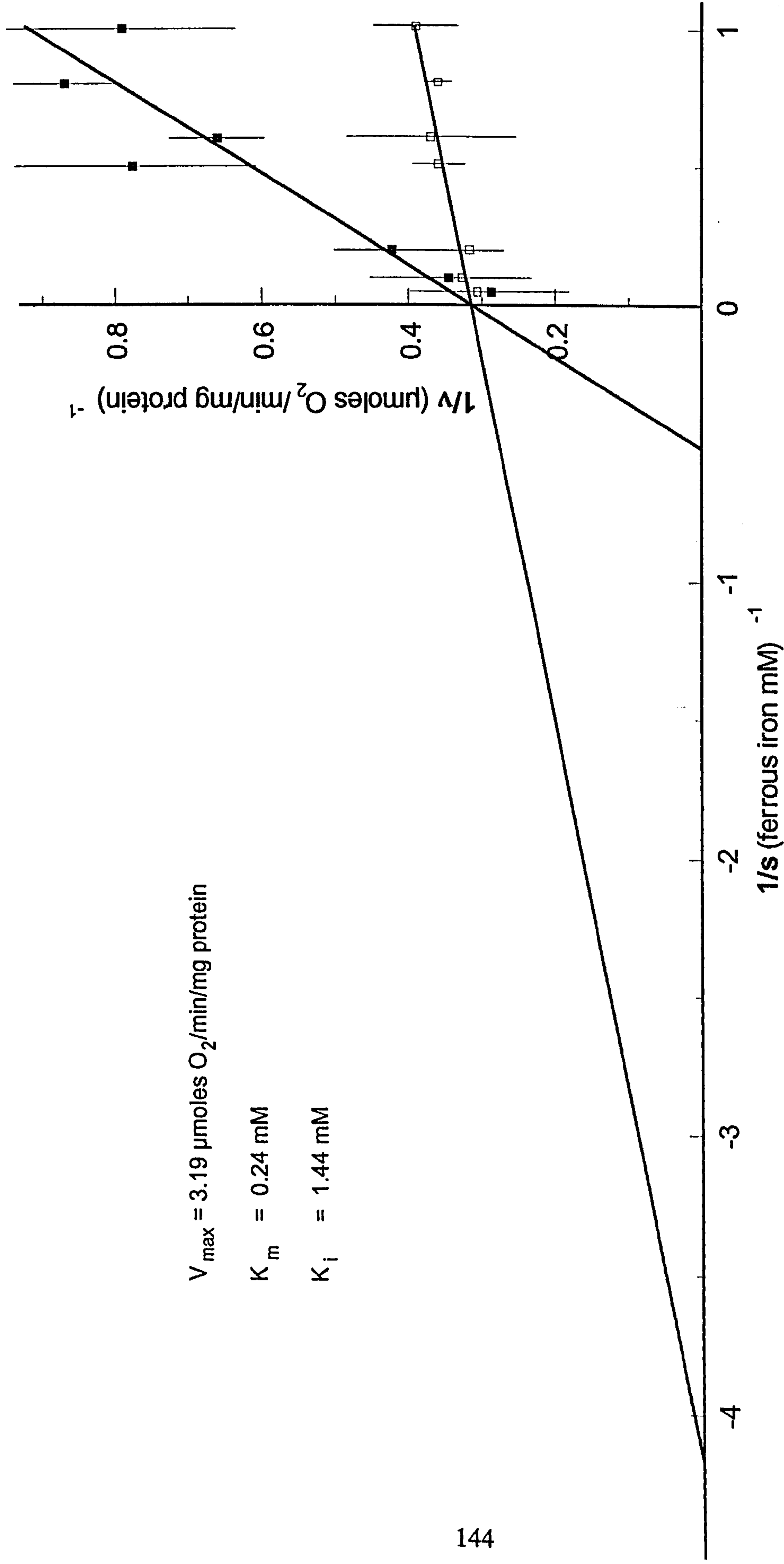


Figure 4.23: Lineweaver-Burk double reciprocal plot for oxygen uptake rates of strain ICP cell suspensions at 45 °C and different ferrous iron concentrations. Cells were grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract at pH 1.7 and 48 °C. Lines were drawn using regression analysis. Bars represent the standard deviation of four values. Oxygen uptake rates are given in the presence (■) and absence (□) of 10 mM ferric iron.

These values for strain ICP are slightly lower than those obtained previously for strain TH3 (Wood *et al.*, 1987), with a $K_m:K_i$ ratio of 1:6 for strain ICP, compared to 1:4 for strain TH3. This high apparent affinity for ferrous iron by these cells was reflected in the rapid growth of strain ICP chemolithoheterotrophically (Figure 4.24). In contrast to this, autotrophically grown strain ICP cells had a K_m of 0.516 mM ferrous iron, and a K_i of 0.403 mM ferric iron, with a resulting ratio of 1:0.78 (Figure 4.25). This indicated that autotrophically grown strain ICP cells had a higher affinity for ferric iron than ferrous iron, a similar result to that obtained with strain ALV (Wood *et al.*, 1987). This higher apparent affinity for ferric iron could explain the poor autotrophic growth of this strain (Fig. 4.24), and the growth inhibition caused by ferric iron (Fig. 4.22).

Chemolithoheterotrophically grown *S. thermosulfidooxidans* cells had a K_m of 0.655 mM ferrous iron and a K_i of 3.6 mM ferric iron (Figure 4.26), whilst autotrophically grown cells had a K_m of 0.245 mM ferrous iron and K_i of 5.67 mM ferric iron (Figure 4.27). *S. thermosulfidooxidans* cells from both growth conditions had much greater affinities for ferrous iron than ferric iron, with $K_m:K_i$ ratios of 1:6 and 1:23 for chemolithoheterotrophic and autotrophic cells respectively. This high affinity for ferrous iron is reflected in the growth curves, which are almost identical during autotrophic and chemolithoheterotrophic growth (Fig 4.24).

All calculations used here were based on the double reciprocal plot of data (Lineweaver and Burk, 1934). This method is known to produce the best-looking straight line for a data set, but can often conceal a poor fit between data and line (Henderson, 1985). To ensure that the results obtained here were not weighted by the data analysis, K_m and K_i values were also calculated from two alternative graphical plots; the v against v/s plot, and the s/v against s plot. The values obtained from each of these plots are given in table 4.3.

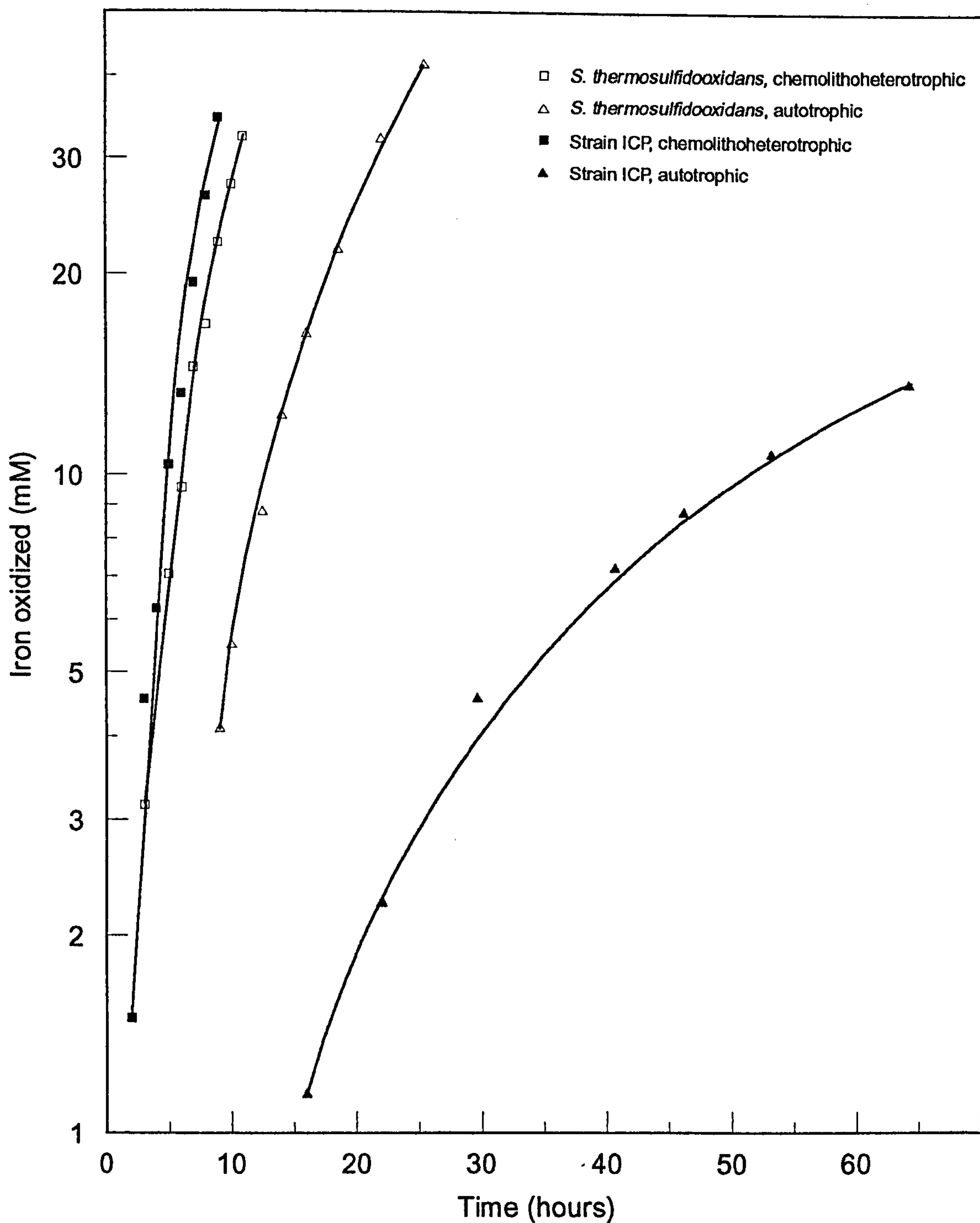


Figure 4.24: Iron oxidation during the growth of two moderately thermophilic iron-oxidizing bacteria at pH 1.7, and 48 °C. Cultures were grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract, and autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air.

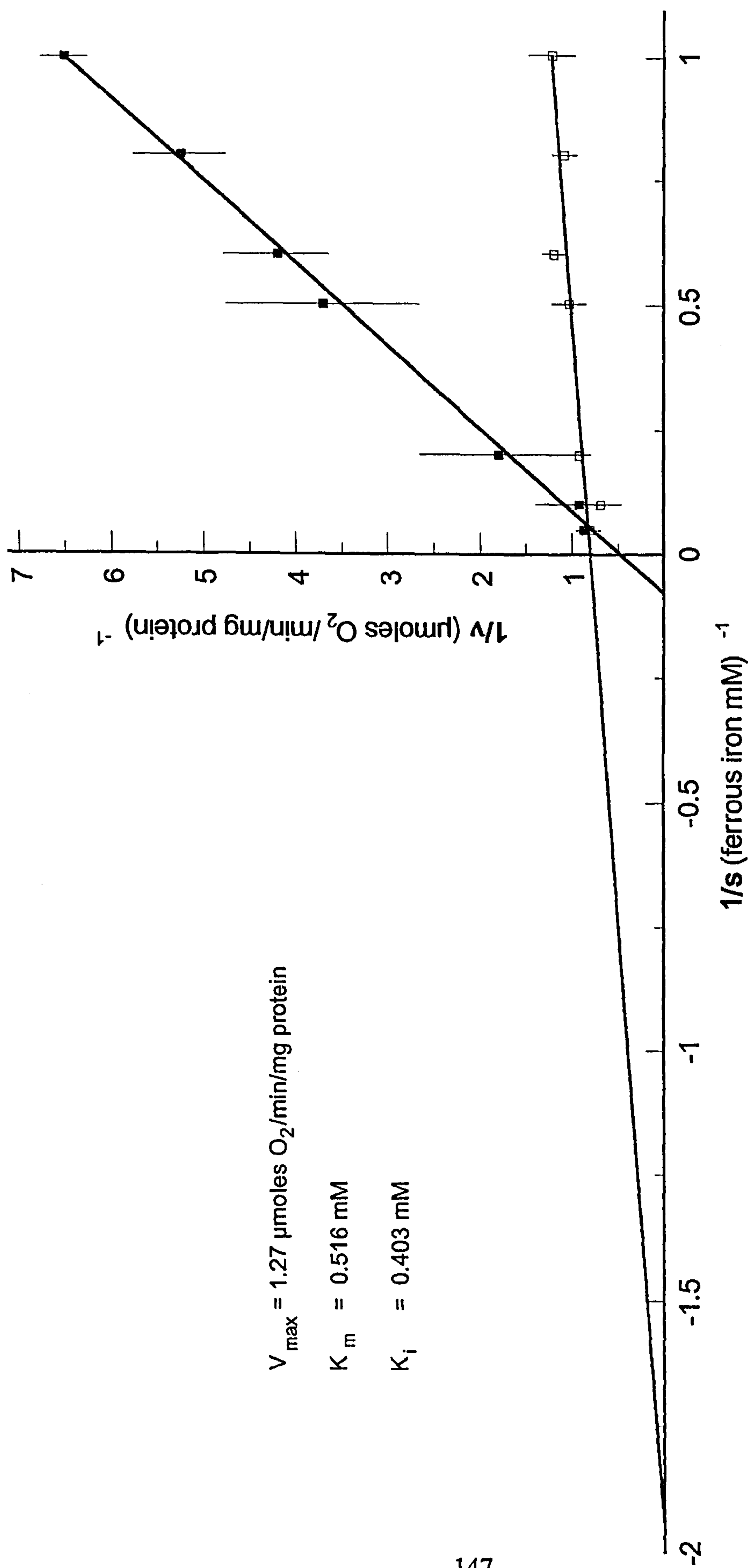


Figure 4.25: Lineweaver-Burk double reciprocal plot for oxygen uptake rates of strain ICP cell suspensions at 45 °C and different ferrous iron concentrations. Cells were grown autotrophically on 50 mM ferrous iron supplemented with 0.05 mM tetrathionate under air, at pH 1.7 and 48 °C. Lines were drawn using regression analysis. Bars represent the standard deviation of four values. Oxygen uptake rates are given in the presence (■) and absence (□) of 10 mM ferric iron.

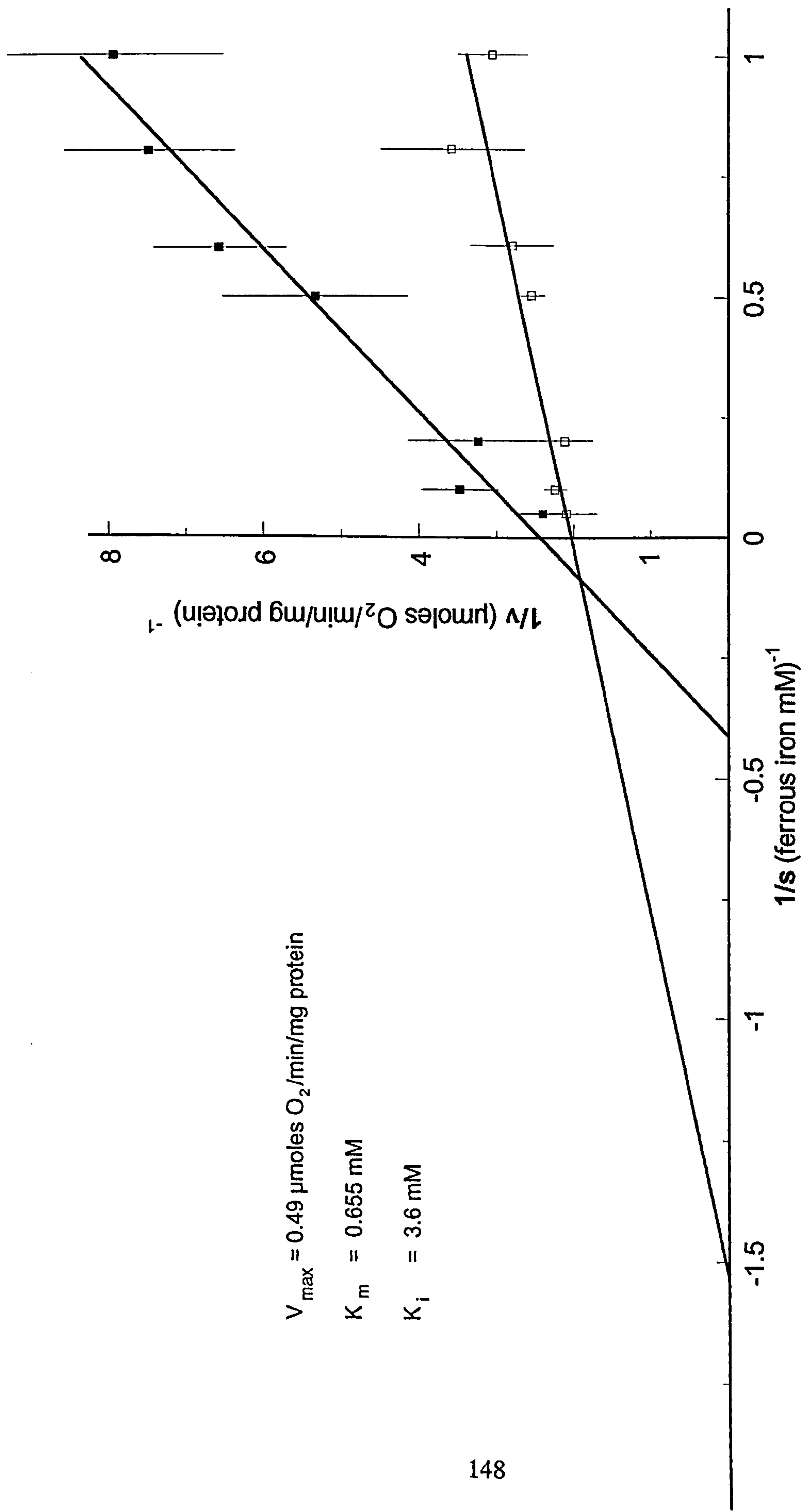


Figure 4.26: Lineweaver-Burk double reciprocal plot for oxygen uptake rates of *S. thermosulfidooxidans* cell suspensions at 45 °C and different ferrous iron concentrations. Cells were grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract at pH 1.7 and 48 °C. Lines were drawn using regression analysis. Bars represent the standard deviation of four values. Oxygen uptake rates are given in the presence (■) and absence (□) of 10 mM ferric iron.

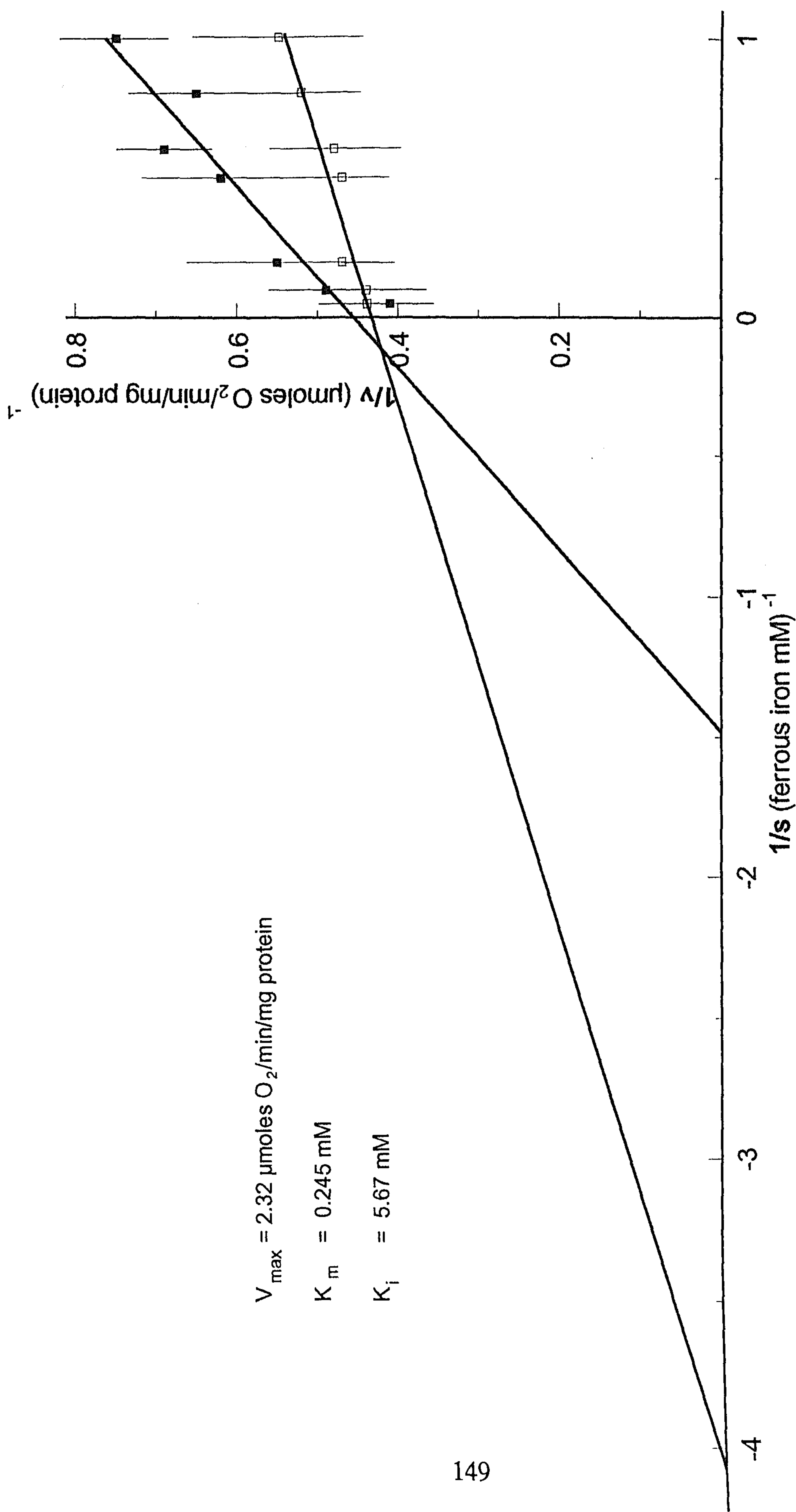


Figure 4.27: Lineweaver-Burk double reciprocal plot for oxygen uptake rates of *S. thermosulfidooxidans* cell suspensions at 45 °C and different ferrous iron concentrations. Cells were grown autotrophically on 50 mM ferrous iron supplemented with 0.05 mM tetrathionate at pH 1.7 and 48 °C, under 1% (v/v) CO₂ in air. Lines were drawn using regression analysis. Bars represent the standard deviation of four values. Oxygen uptake rates are given in the presence (■) and absence (□) of 10 mM ferric iron.

Although there was some variation in the values obtained from the three graphical plots, the relationship between K_m and K_i remained the same for each organism. All three plots for autotrophically grown strain ICP gave a higher affinity for ferric iron than ferrous iron (although in two of the three cases K_m and K_i values were almost identical). Chemolithoheterotrophically grown strain ICP cells, and both autotrophic and chemolithoheterotrophic *S. thermosulfidooxidans* cells had higher affinities for ferrous iron than ferric iron. The s/v against s plot is believed to be the best graphical method for K_m estimations (Henderson, 1985), although the choice of method is still largely a matter of personal preference.

	Graphical plot:								
Strain and growth conditions	1/s	Vs	1/v	s/v	Vs	s	v	Vs	v/s
	K _m		K _i	K _m		K _i	K _m		K _i
Strain ICP, autotrophic ^a	0.52		0.40	0.58		0.56	0.65		0.63
Strain ICP, chemolithoheterotrophic ^b	0.24		1.44	0.46		1.71	0.37		1.54
<i>S. thermosulfidooxidans</i> , autotrophic ^a	0.25		5.67	0.24		2.24	0.27		4.36
<i>S. thermosulfidooxidans</i> , chemolithoheterotrophic ^b	0.66		3.6	0.55		2.09	0.38		2.02

^a Autotrophic growth on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air.

^b Chemolithoheterotrophic growth on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract.

Table 4.3: Comparison of apparent K_m and K_i values for ferrous and ferric iron respectively by cell suspensions of moderately thermophilic acidophilic iron-oxidizing bacteria. Values (in mM) were derived from three alternative linear forms of the Michaelis-Menton equation.

These results suggest that the poor autotrophic growth of strain ICP is a result of a higher affinity for ferric iron than ferrous iron. Good chemolithoheterotrophic growth occurs because in these conditions the cells have a much lower affinity for ferric iron. An increase in affinity for ferric iron during autotrophic growth is not observed with *S. thermosulfidooxidans* cells, consequently the growth curve is not affected.

4.5 Discussion.

Attempts to isolate a single bacterial type from the Icelandic mixed culture capable of autotrophic growth under air to the extent seen by the mixed culture failed. A culture capable of good growth under air was obtained (ICC). Various methods, including the use for Percoll density gradients, indicated this enrichment culture consisted of at least two distinct cell types, a typical *S. thermosulfidooxidans* DNA:DNA hybridisation group organism and a novel cell type.

Difficulties in isolating this novel cell type were associated with poor growth on solid substrates, but continuous sub-culturing of the ICC mixed culture autotrophically under air, eventually resulted in single colonies of the novel cell type. The reason for this sudden ease of plating after so many failures is not known. The most likely explanation is that a good colony forming strain was selected for during continuous sub-culturing.

Characterisation of the novel isolate (strain ICP) indicated that it was a member of the third DNA:DNA hybridisation group of moderately thermophilic iron-oxidizing bacteria (see section 3.2). It was very closely related to the only other member of this group, strain TH3, both morphologically and physiologically. The only apparent difference between the two isolates being the tendency of strain TH3 to form a filamentous morphology during chemolithoheterotrophic growth.

Autotrophic growth of strain ICP alone did not explain the levels of iron oxidation achieved by the ICC mixed culture. The presence of a second iron-oxidizing organism was essential for good iron oxidation rates. The second iron-oxidizing bacterium within the ICC culture was a *S. thermosulfidooxidans* DNA:DNA hybridisation group organism. Other isolates grown in co-culture with strain ICP gave similar levels of iron oxidation to the ICC culture. Isolates from both the *S. thermosulfidooxidans* group, and NAL group gave good rates of iron oxidation in co-culture with strain ICP. Strain ICP was believed to be essential in these cultures, because of its high CO₂ utilisation capacities (see chapter 5).

Autotrophic growth of strain ICP was not enhanced by the addition of CO₂ levels above those in air, unlike most other moderately thermophilic iron-oxidizers. Poor autotrophic growth, even at enhanced CO₂ levels appears to be a result of a greater affinity for the end product of oxidation, ferric iron, than the substrate, ferrous iron. It is thought that when strain ICP is cultured in iron containing media, rapid iron oxidation is seen initially because all the iron present is ferrous iron. However, ferric iron is produced during iron oxidation and cell growth, and rapidly reaches inhibitory levels, resulting in the typical growth curve displayed by strain ICP. Similar iron affinities and poor autotrophic growth were previously reported with strain ALV (Wood *et al.*, 1987).

This iron oxidation kinetic data explains the growth data obtained with strain ICP, but the question of why the addition of yeast extract should have such a marked effect on apparent iron affinities of strain ICP cells remains. There are two possible explanations:

1. The growth of strain ICP on ferrous iron plus an organic compound such as yeast extract compared to that on ferrous iron in the presence of a single C_i compound (i.e. CO₂) may affect the cell surface, and thus alter the whole cell affinity for iron.
2. The different affinities shown by cells from different growth conditions may simply be a reflection of the health of the cells. Cells grown in the presence of a rich carbon compound like yeast extract may be healthier than those grown on CO₂ alone.

Only by studying the pathway of iron oxidation in these organisms under the different conditions will the correct theory be discovered, as the data presented here is only an indication of the whole cell apparent affinity and not the actual enzyme kinetics.

The ability of the ICC culture to grow under air, therefore, appears to be a consortium effect based on the interactions between two main cell types; strain ICP an efficient CO₂ utiliser, and strain ICH an efficient autotrophic iron-oxidizer. It is believed that strain ICP initiates autotrophic growth under air, but the second cell type is essential to maintain high iron oxidation rates. The second cell type probably utilises carbon fixed by strain ICP for its growth requirements, but the exact interaction between the isolates is still not clearly understood. Strain ICH may take over iron-oxidation completely during the second half of the mixed cultures growth phase, as strain ICP becomes inhibited by the increasing ferric iron levels. Alternatively strain ICP may in turn utilise fixed carbon from strain ICH after an initial short autotrophic growth phase. Such a switch to chemolithoheterotrophic growth might be sufficient to lower the cell's affinity for ferric iron, enabling further iron oxidation by strain ICP. Thus iron oxidation by both organisms would continue to stationary phase, with each organism providing fixed carbon for the other to utilise. Whatever the relationship between these two isolates, both are clearly essential for good oxidation rates.

Chapter 5:

CO₂ Utilisation Studies.

5.1: Introduction.

Many micro-organisms fix carbon dioxide as their sole source of carbon, using a variety of pathways, the main one being the Calvin cycle or reductive pentose phosphate pathway (Tabita, 1988). The first enzyme of CO₂ fixation in this cycle is ribulose biphosphate carboxylase/oxygenase (RuBisCO). This enzyme exhibits a poor affinity for CO₂, its substrate. Intact cells, however, have a much greater affinity for CO₂ than the isolated enzyme (Miller *et al.*, 1984). This greater affinity of whole cells is achieved by accumulating high levels of intracellular C_i. These elevated levels cannot be accounted for by simple diffusion across the membrane, indicating an active C_i transport system. (Kaplan *et al.*, 1994).

The carbon concentrating mechanisms (CCMs), believed to be responsible for active transport, are known to respond to low external CO₂ levels by increasing in activity (Coleman, 1991), but the actual mechanisms of transport are still not understood. Little data on CCMs in chemolithotrophic bacteria is available. An inducible, high affinity CCM has been demonstrated in *Thiobacillus neapolitanus* (Holthuijzen *et al.*, 1987). This organism responds to intracellular C_i limitation by the induction of a high affinity CCM, enabling growth on thiosulphate under air. Active CO₂ transport required the presence of an exogenous energy source, and was dependent on the membrane potential. *Thiobacillus versutus* lacks such a high affinity CCM, and despite similar RuBisCO activities to *T. neapolitanus*, does not grow on thiosulphate under air (Karagouni and Kelly, 1989).

Comparison of moderate thermophile strains BC1 and ALV to *Thiobacillus ferrooxidans* indicated that the inability of strains BC1 and ALV to grow under air levels of CO₂ was a result of the lack of an inducible, high affinity CCM, rather than any major differences in the enzymes of CO₂ fixation (MacLean, 1993). In addition these two organisms were unable to take up CO₂ immediately after harvesting of the cells. This lack of CO₂ uptake was restored by incubation of the cells at 45 °C for 4 hours in the presence the energy source, ferrous iron.

The activity of RuBisCO within an organism is used as an indication of CO₂ fixation via the Calvin cycle. The typical form of this enzyme is a hexadecameric molecule consisting of 8 large (56 kDa), and 8 small (15 kDa) subunits (Tabita, 1994). Some *Rhodospirillum* species possess a second form of RuBisCO, consisting of a dimer of large subunits only (Nargang *et al.*, 1984). These two forms of the enzyme are distinct both immunologically and in their primary protein sequences (Gibson and Tabita, 1985; English *et al.*, 1992). The structure of this enzyme is, however highly conserved, with hexadecameric forms from all sources showing >70% homology.

5.2: CO₂ uptake studies.

5.2.1: CO₂ uptake by culture ICC.

A mixed moderately thermophilic culture, ICC, was found to be capable of good autotrophic growth on ferrous iron under air levels of CO₂ (see section 4.2.1). This culture was investigated for the presence of an inducible high affinity CCM.

1 l of culture ICC was grown autotrophically on 50 mM ferrous iron in 2 l shaken flasks, gassed with either air or 1% (v/v) CO₂ in air. Cells were removed during the phase of maximum iron oxidation, and assayed for CO₂ uptake over a 5 minute time course by following radioactive label incorporation, after the addition of ¹⁴C-labelled bicarbonate. The bicarbonate concentrations added corresponded to a range of CO₂ concentrations between 0.04% to 0.47% (v/v) CO₂ in air.

A time course was established for CO₂ uptake at each CO₂ concentration for cells grown under 1% (v/v) CO₂ in air (Figure 5.1), and for cells grown under air (Figure 5.2). The maximum rates of ¹⁴C-label incorporation were estimated from these time courses and converted to nmol CO₂/min/mg protein. These maximal rates were then plotted against the respective CO₂ concentrations in the assay (Figure 5.3).

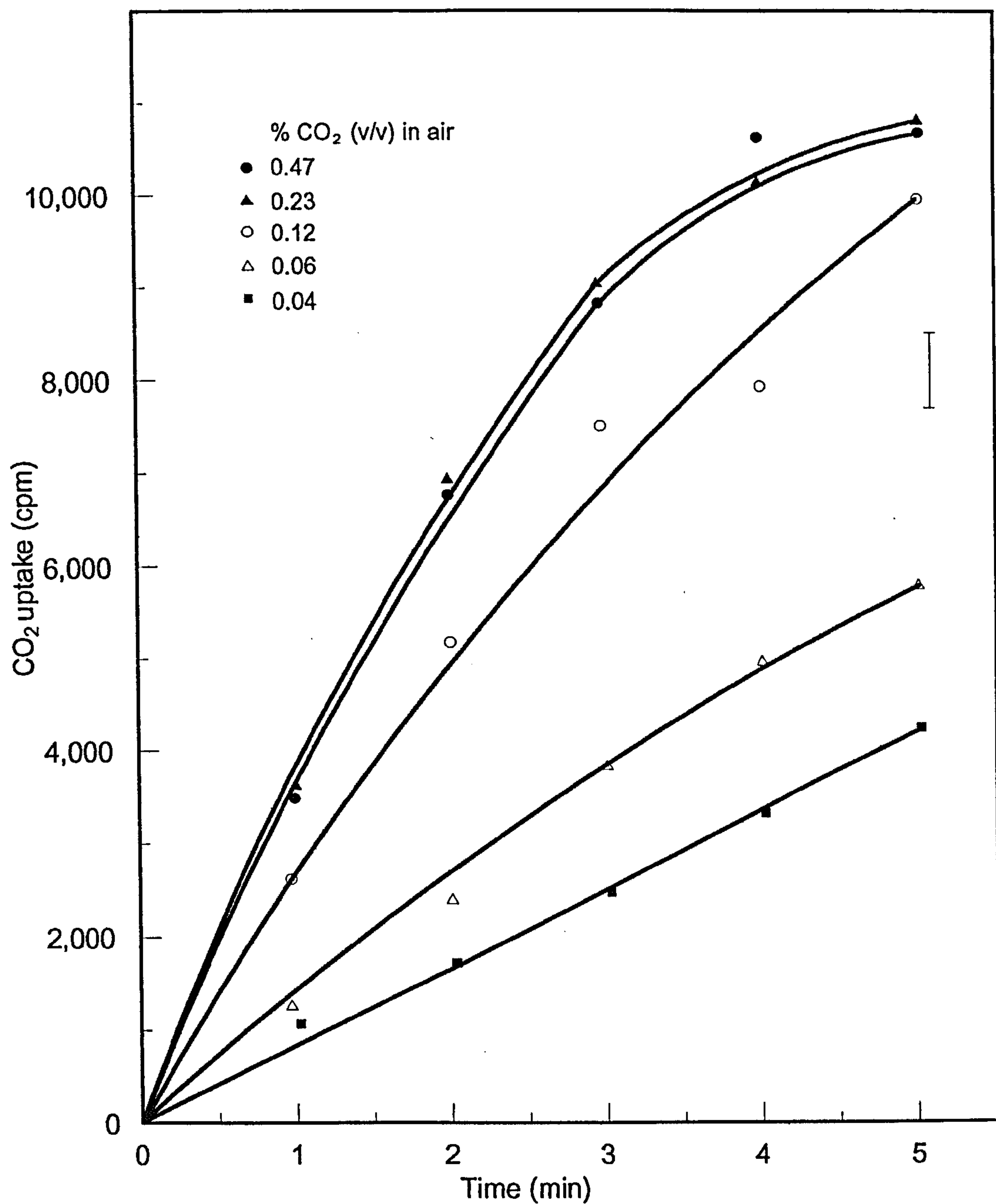


Figure 5.1: CO₂ uptake at 45°C, and various CO₂ concentrations by autotrophically grown culture ICC. Cells were grown on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48°C, under 1% CO₂ (v/v) in air. The maximum uptake rates at each assay CO₂ concentration were converted to nmol CO₂/min/mg protein (see section 2.12), and then plotted in Figure 5.3. The bar represents maximum standard error.

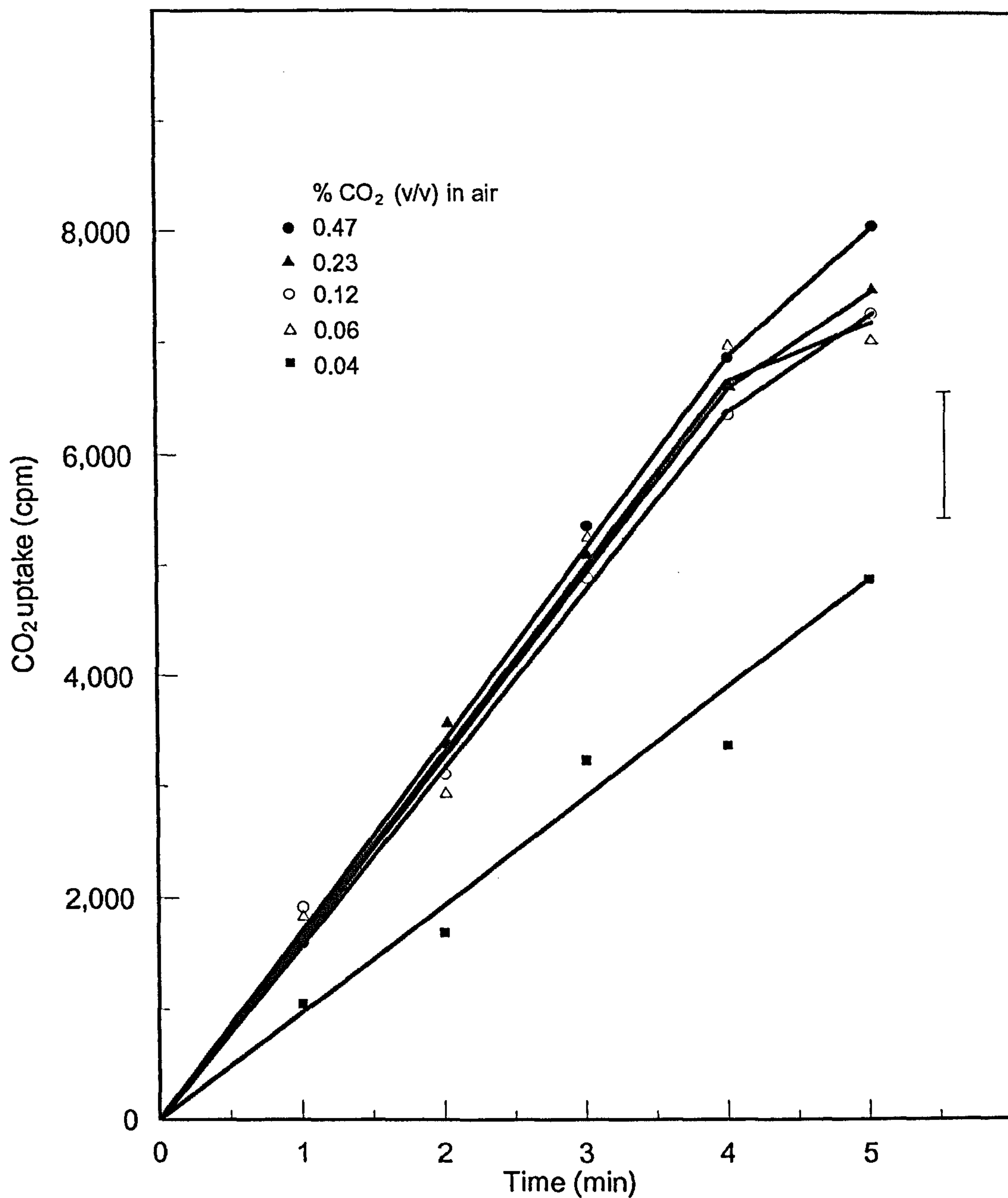


Figure 5.2: CO₂ uptake at 45°C, and various CO₂ concentrations by autotrophically grown culture ICC. Cells were grown on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48°C, under air. The maximum uptake rates at each assay CO₂ concentration were converted to nmol CO₂/min/mg protein (see section 2.12), and then plotted in Figure 5.3. The bar represents maximum standard error.

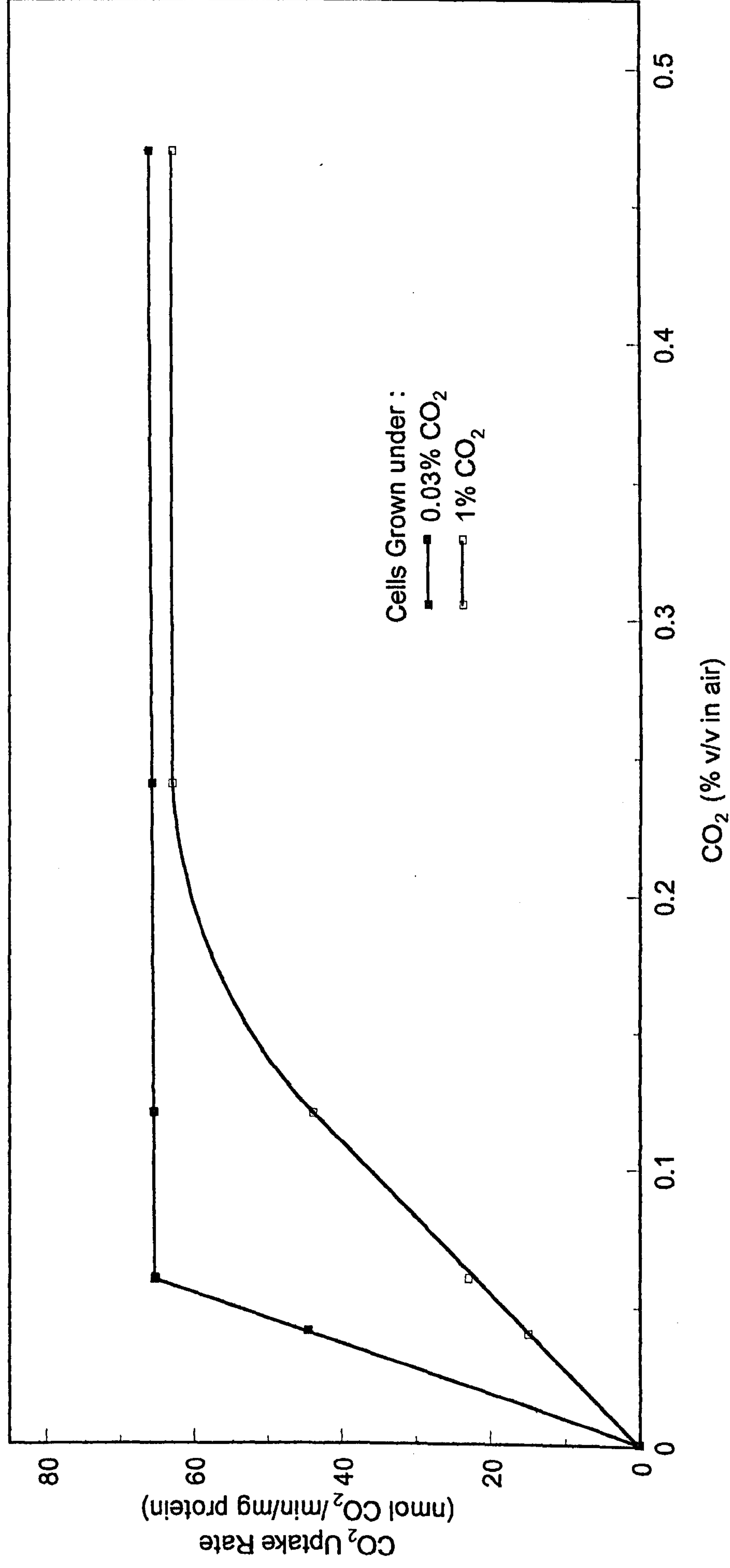


Figure 5.3: Maximal CO₂ uptake rates for culture ICC at 45 °C, and a range of CO₂ assay concentrations. Culture ICC was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48 °C, under air or 1% CO₂ (v/v) in air.

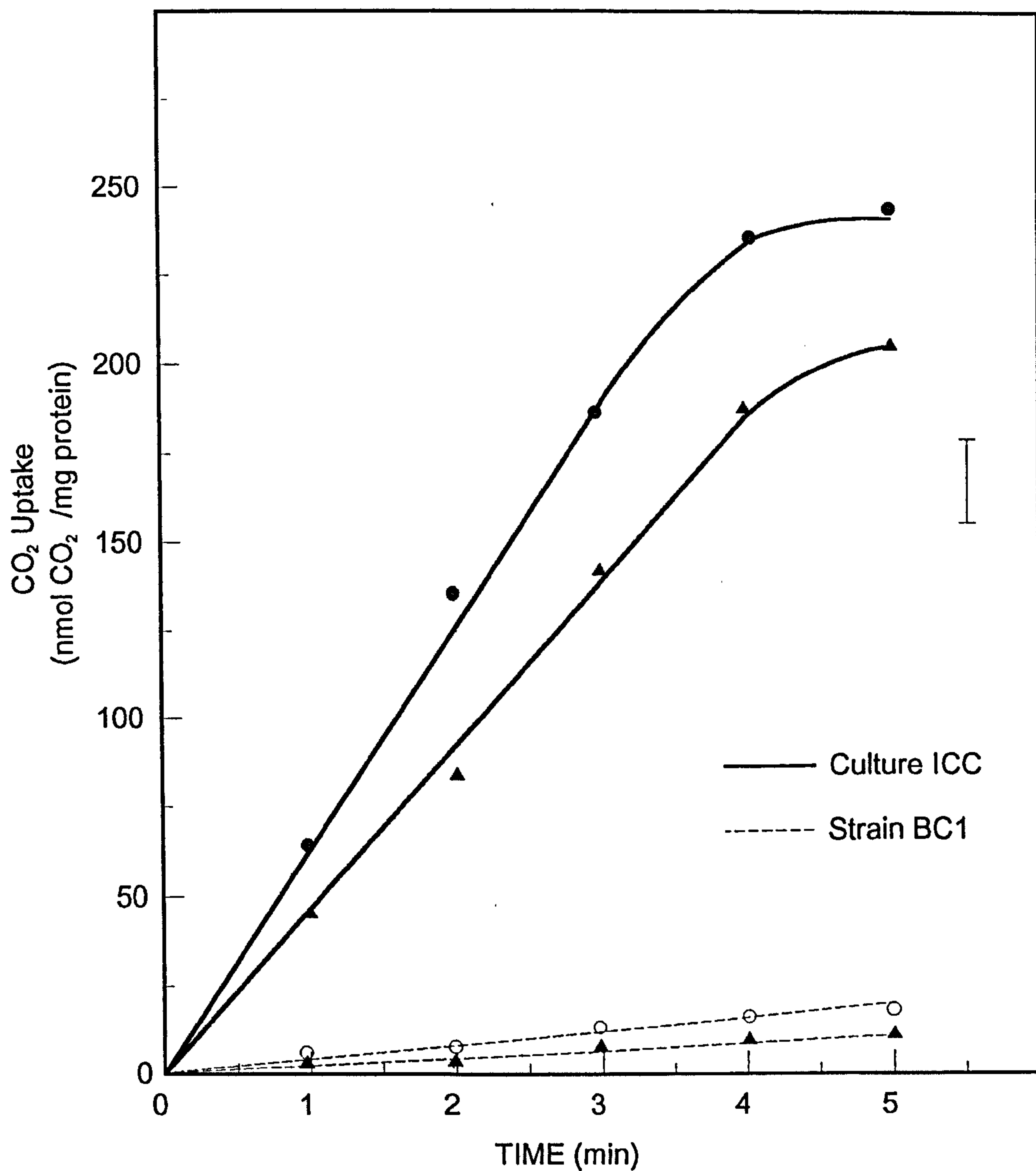
Similar maximal transport rates of between 60 and 65 nmol CO₂/min/mg protein were obtained with 1% (v/v) CO₂ grown cells and air grown cells (Fig. 5.3). Comparable maximal rates were previously obtained with strain BC1 and *T. ferrooxidans* (MacLean, 1993). The ICC cells grown under air, however, reached this maximal transport rate at a much lower CO₂ assay concentration (0.05% (v/v) CO₂ in air) than cells grown under 1% (v/v) CO₂ in air (0.24% (v/v) CO₂ in air), indicating that a high affinity CCM was induced by growth under air.

Air-grown cells were capable of a maximal transport rate of 30 nmol CO₂/min/mg protein (approximately 50% saturation of the transport system), at a CO₂ assay concentration of 0.03% (v/v), the concentration of CO₂ found in air. This uptake rate has previously been shown to be insufficient for the autotrophic growth of strain BC1 (MacLean, 1993). However, the presence of an organism within the mixed culture not contributing to CO₂ uptake would result in the overestimation of the protein that should be included in the calculation to determine the true specific uptake rate. Therefore, the true specific uptake rate could only be estimated once the two organisms within the mixed culture had been separated, and the nature of their association discovered (see chapter 4).

5.2.2: Requirement of re-suspended cells of culture ICC for a regeneration period.

Moderate thermophile strains BC1 and ALV had an obligate regeneration requirement, following harvesting and re-suspension, before CO₂ transport would occur (MacLean, 1993). The moderately thermophilic ICC culture was examined for a regeneration requirement following harvesting of the cells.

Cells of culture ICC and strain BC1 were harvested by centrifugation, and washed. The washed cell suspensions were allowed to equilibrate at 45 °C. Half the cells were then assayed immediately for their CO₂ transport capacities (Figure 5.4), whilst the rest were incubated for 4 hours in the presence of ferrous iron, and then assayed for CO₂ transport (Figure 5.5).



- ▲ Cells Grown Autotrophically with 1% (v/v) CO₂ in air
- Cells Grown Autotrophically with 0.03% (v/v) CO₂ in air
- Cells Grown Chemolithoheterotrophically on Fe²⁺/ Yeast Extract.

Figure 5.4: CO₂ uptake by re-suspended cells of culture ICC and strain BC1, in the presence of 50 mM ferrous iron and an atmosphere of 0.1% (v/v) CO₂ in air, at 45 °C. Cells were assayed immediately upon harvesting and re-suspended in medium (pH 1.7). The bar represents maximum standard error.

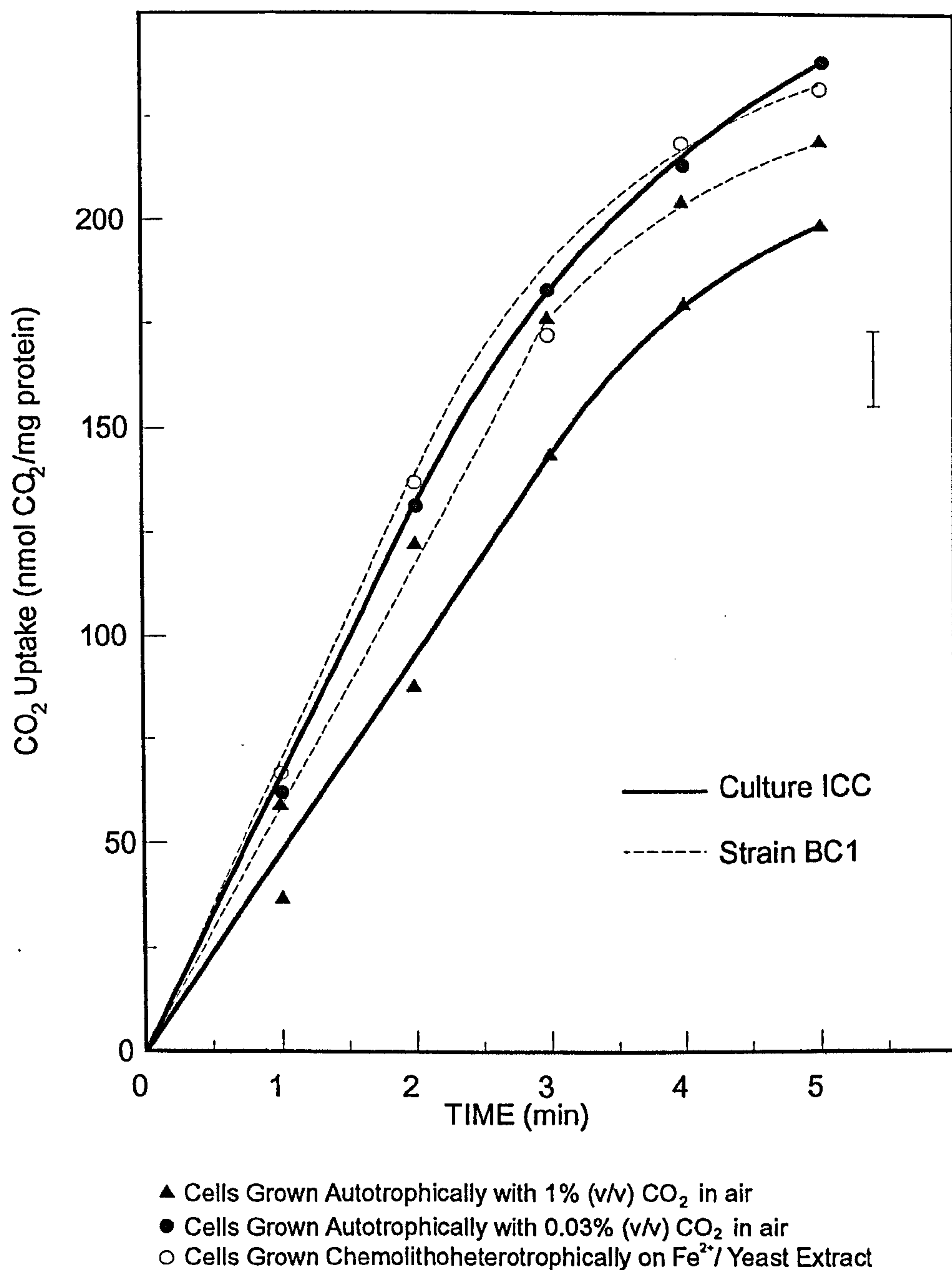


Figure 5.5: CO₂ uptake by re-suspended cells of culture ICC and strain BC1, in the presence of 50 mM ferrous iron and an atmosphere of 0.1% (v/v) CO₂ in air, at 45 °C, after a 4 hour incubation period. Cells were harvested, re-suspended in medium (pH 1.7), and incubated at 45°C in a shaking water bath, with 50 µl additions of 1 M FeSO₄·7H₂O made every 30 min. Cell suspensions were then assayed as previously. The bar represents maximum standard error.

^{14}C -label was rapidly incorporated by ICC cells immediately after re-suspension, at rates of 45 nmol CO_2 /min/mg protein for cells grown under 1% (v/v) CO_2 in air, and 65 nmol CO_2 /min/mg protein for air-grown cells (Fig. 5.4). This indicated that the ICC culture did not require a regeneration period in order to achieve optimal CO_2 transport. In contrast strain BC1 cells were only capable of a very poor rate of CO_2 uptake (5 nmol CO_2 /min/mg protein) immediately upon re-suspension (Fig. 5.4). The full transport capacity of strain BC1 was restored following a four hour regeneration period (Fig. 5.5).

The ability of the ICC culture to grow well autotrophically under air therefore appears to be a result of the induction of a high affinity CO_2 uptake system (Fig. 5.3). The culture also differed from previously studied moderate thermophiles in that re-suspended cells did not require regeneration before showing maximum CO_2 transport (Fig. 5.4). The specific uptake rates achieved by culture ICC grown under air appeared too low to sustain purely autotrophic growth, suggesting that one of the two organisms within the culture was scavenging fixed carbon. Separation of the major organisms from this mixed culture, therefore, had to be achieved before further investigations were conducted.

5.2.3: CO_2 Uptake by pure isolates from the ICC mixed culture.

Upon separation of the two distinct cell types within the mixed culture it was found that one isolate, strain ICH, was a *S. thermosulfidooxidans* DNA:DNA hybridisation group organism (see chapter 4). This isolate grew poorly on ferrous iron under air levels of CO_2 . The second isolate, strain ICP, was a TH3 DNA:DNA hybridisation group organism (see chapter 4), and grew reasonably well on ferrous iron under air levels of CO_2 (see Fig. 4.20).

Both isolates were assayed for a high affinity CO_2 uptake system. Cultures were grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate,

and gassed with either 1% (v/v) CO₂ in air, or air. Mid-exponential cells were assayed for CO₂ uptake as described previously (see section 5.2.1). The poor autotrophic growth yield achieved by strain ICP (see section 4.4.2) resulted in uptake levels by growing cells that were too small to be accurately measured. Therefore, assays with strain ICP had to be carried out using harvested and re-suspended cells.

¹⁴C-label was rapidly incorporated by strain ICP grown under 1% (v/v) CO₂ in air, and under air alone (Figures 5.6 and 5.7). The maximal CO₂ uptake rates for each CO₂ assay concentration were calculated from these time courses, and plotted against the respective CO₂ assay concentrations (Figure 5.8).

The results clearly indicated that strain ICP possesses an inducible high affinity CCM that operates when cells are grown under air levels of CO₂ (Fig. 5.8). This high affinity uptake system had a similar maximal transport rate to that seen previously with the mixed culture (60 nmol CO₂/min/mg protein). At an assay concentration of 0.03% (v/v) CO₂ in air, air-grown strain ICP cells were capable of a maximal transport rate of 45 nmol CO₂/min/mg protein, approximately 75% of the maximal rate. This value is greater than that observed with the ICC mixed culture, and is similar to the values shown previously to support growth under air (MacLean, 1993). Cells grown at enhanced CO₂ levels showed low affinity uptake similar to that seen previously with the mixed culture. It was also noted that harvested and re-suspended cells of strain ICP, in common with the mixed culture, did not require a period of regeneration before CO₂ uptake occurred. Strain ICH showed identical CO₂ uptake to strain BC1 (data not shown); only a low affinity uptake system even after growth under air.

The possession of an inducible high affinity CCM by strain ICP appears to be the basis of the mixed culture's ability to grow under air levels of CO₂. The essential role of the second organism (strain ICH) in efficient oxidation of iron by the mixed culture is thought to be related to the poor affinity of strain ICP for ferrous iron (see section 4.4.3).

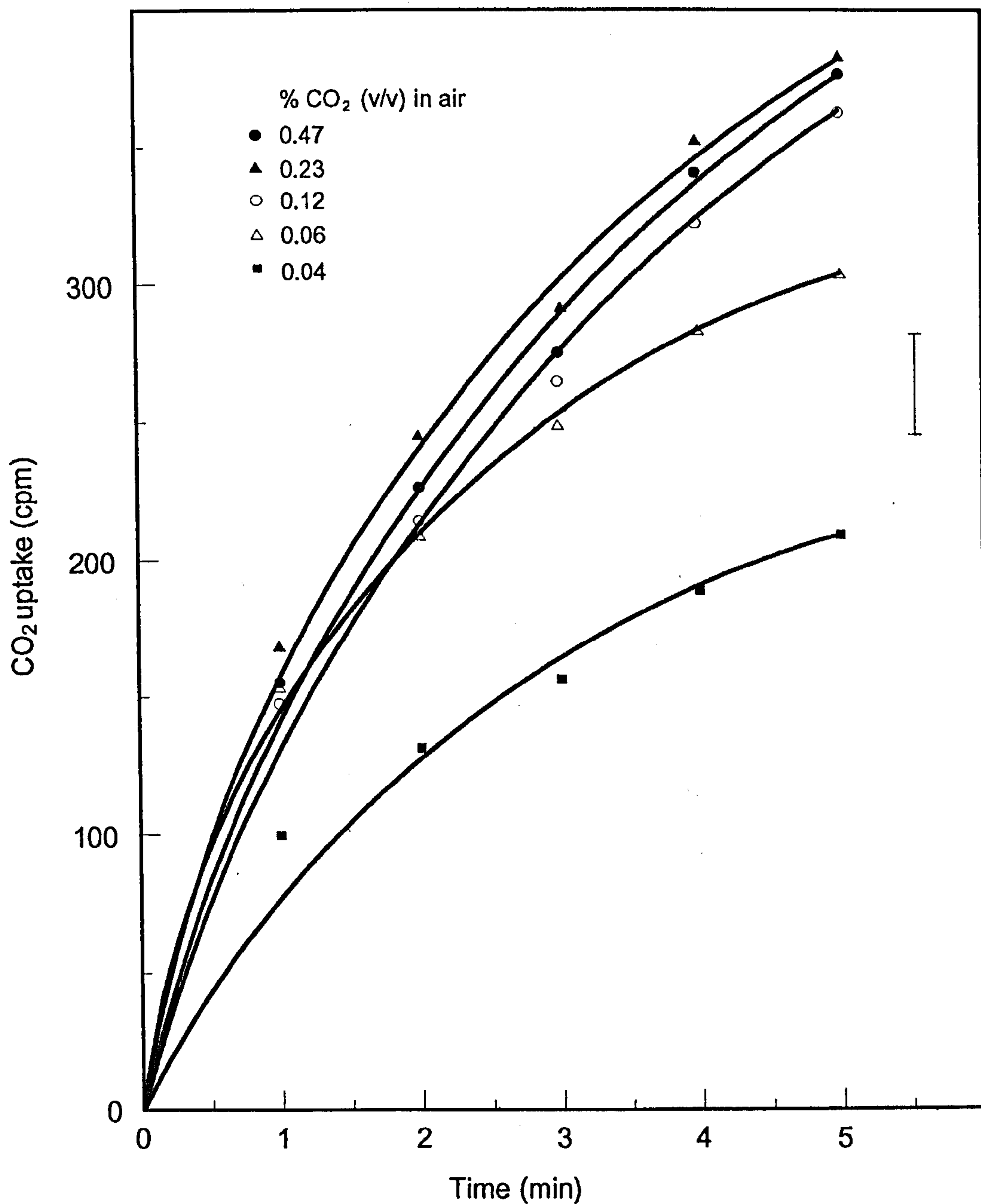


Figure 5.6: CO₂ uptake at 45 °C, and various CO₂ concentrations by autotrophically grown strain ICP. Cells were grown on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48 °C, under 1% (v/v) CO₂ in air. The maximum uptake rates at each assay CO₂ concentration were converted to nmol CO₂/min/mg protein (see section 2.12) and then plotted in Figure 5.8. The bar represents maximum standard error.

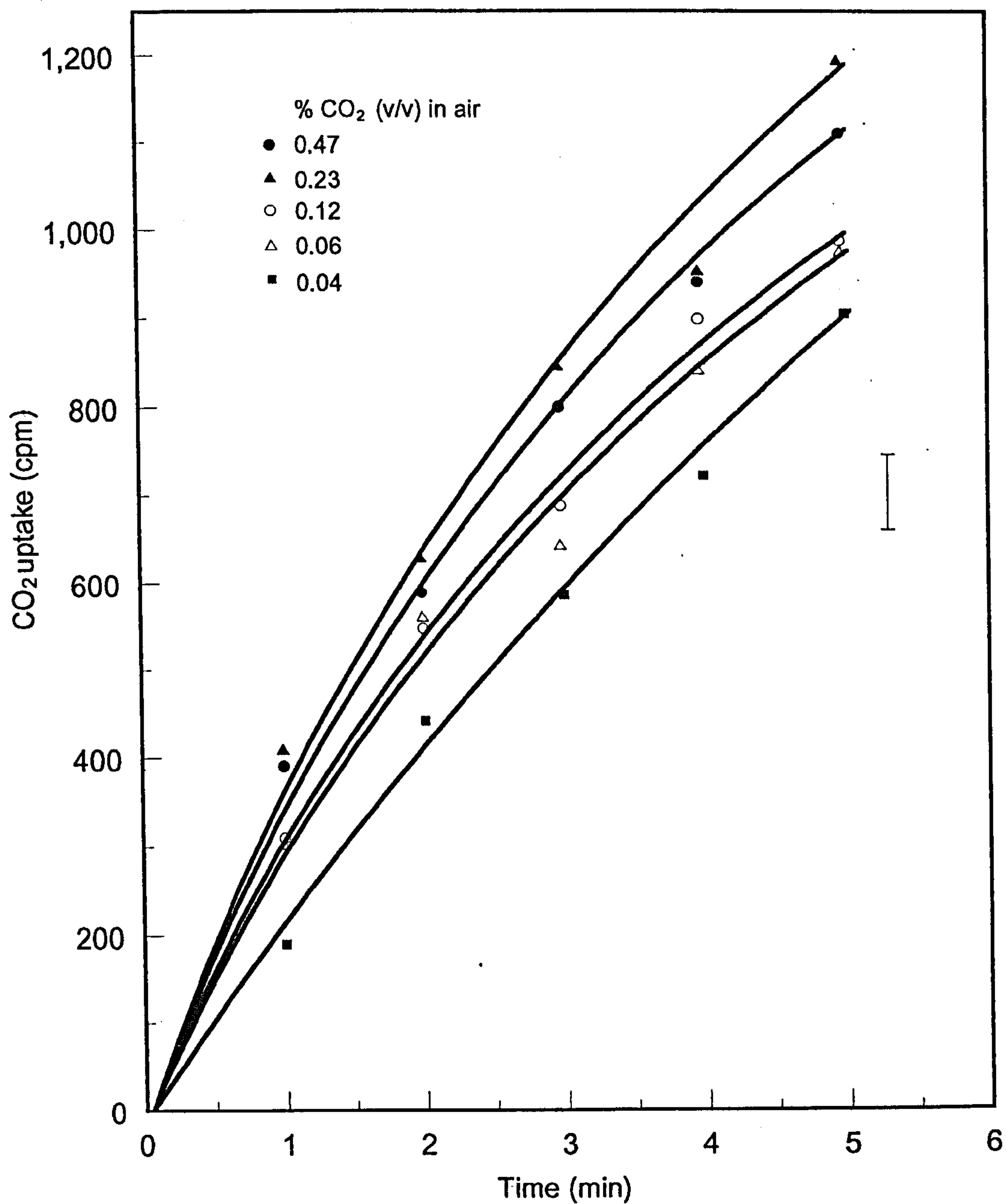


Figure 5.7: CO₂ uptake at 45°C, and various CO₂ concentrations by autotrophically grown strain ICP. Cells were grown on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48°C, under air. The maximum uptake rates at each assay CO₂ concentration were converted to nmol CO₂/min/mg protein (see section 2.12) and then plotted in Figure 5.8. The bar represents maximum standard error.

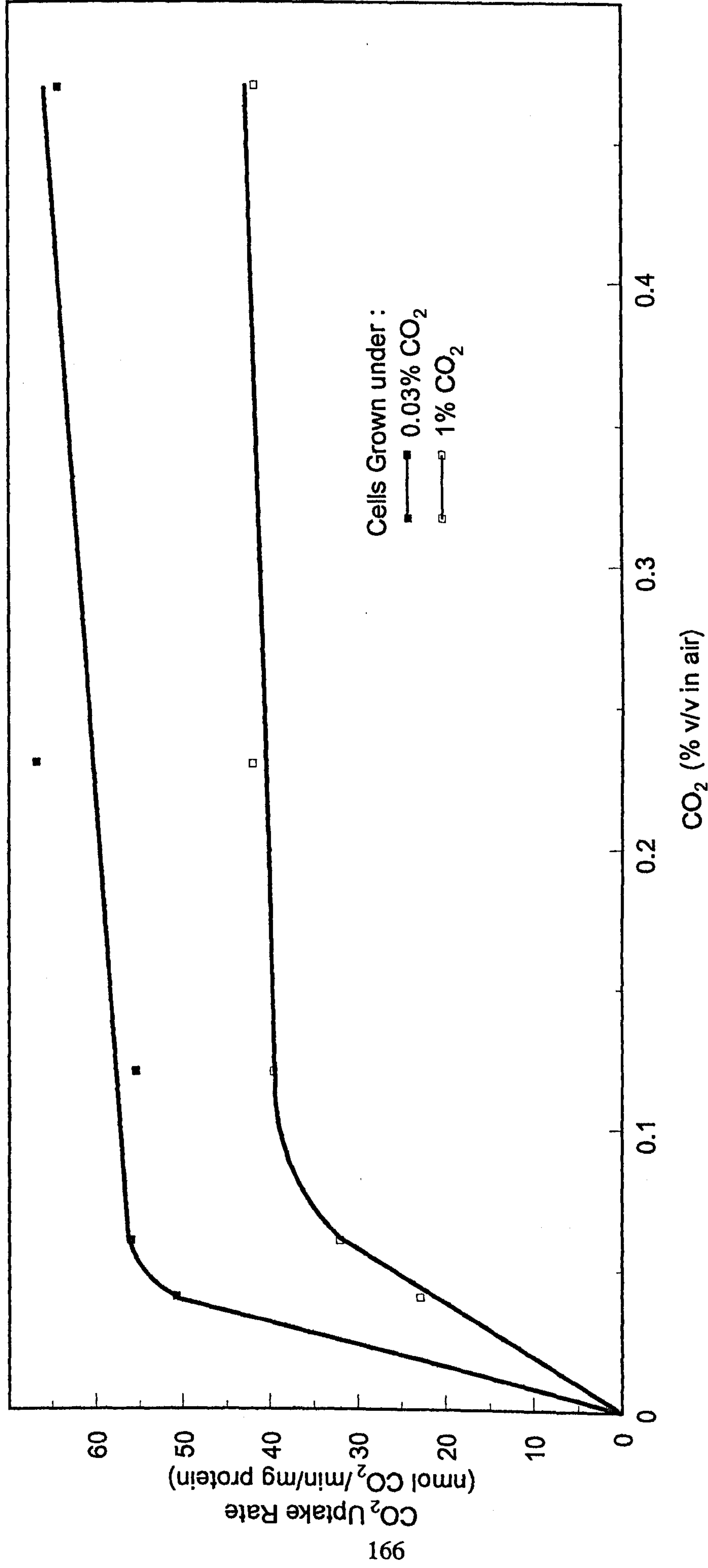


Figure 5.8: Maximal CO₂ uptake rates for strain ICP at 45°C, and a range of CO₂ assay concentrations. Strain ICP was grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, at 48°C, under air or 1% CO₂ (v/v) in air.

5.3: CO₂ fixation studies using strain ICP.

The key role of strain ICP in CO₂ utilisation by the ICC mixed culture was clearly demonstrated in the previous experiments, but the method of CO₂ fixation by strain ICP was not known. The most common pathway of CO₂ fixation is via the Calvin cycle, but alternative pathways exist (Fuchs, 1989). The activity of RuBisCO during autotrophic growth of moderate thermophile strains BC1 and ALV has been demonstrated (Wood and Kelly, 1985). However, strain ICP is not closely related to these isolates (see section 4.3), and so the presence of RuBisCO in strain ICP could not be inferred from activity in strains BC1 and ALV.

5.3.1: Permeabilised whole cell assay for RuBisCO.

Autotrophically grown cells of strain ICP and BC1 were assayed for the presence of RuBisCO using a permeabilised whole cell method (see section 2.13). Cultures were grown on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate at 48 °C, under 1% (v/v) CO₂ in air. Assays were initiated by the addition of ribulose-1,5-bisphosphate, or water as a control. Samples were taken every 5 minutes, for 30 minutes, and activity assayed by the incorporation of ¹⁴C-label. *E. coli* was included as a negative control.

Figure 5.9 shows ¹⁴C-label incorporation fixed during triplicate assays. Both strains BC1 and ICP displayed rapid incorporation of labelled carbon indicating RuBisCO activity. Specific enzyme activities for strains BC1 and ICP were 52.25 ± 0.96 and 10.65 ± 0.63 nmol CO₂/min/mg dry weight respectively. These values are of the right order of magnitude to support CO₂-dependent growth (Wood and Kelly, 1985).

The RuBisCO activities observed clearly indicate that strain ICP utilises CO₂ via the Calvin cycle, in common with the other moderately thermophilic iron-oxidizers studied previously.

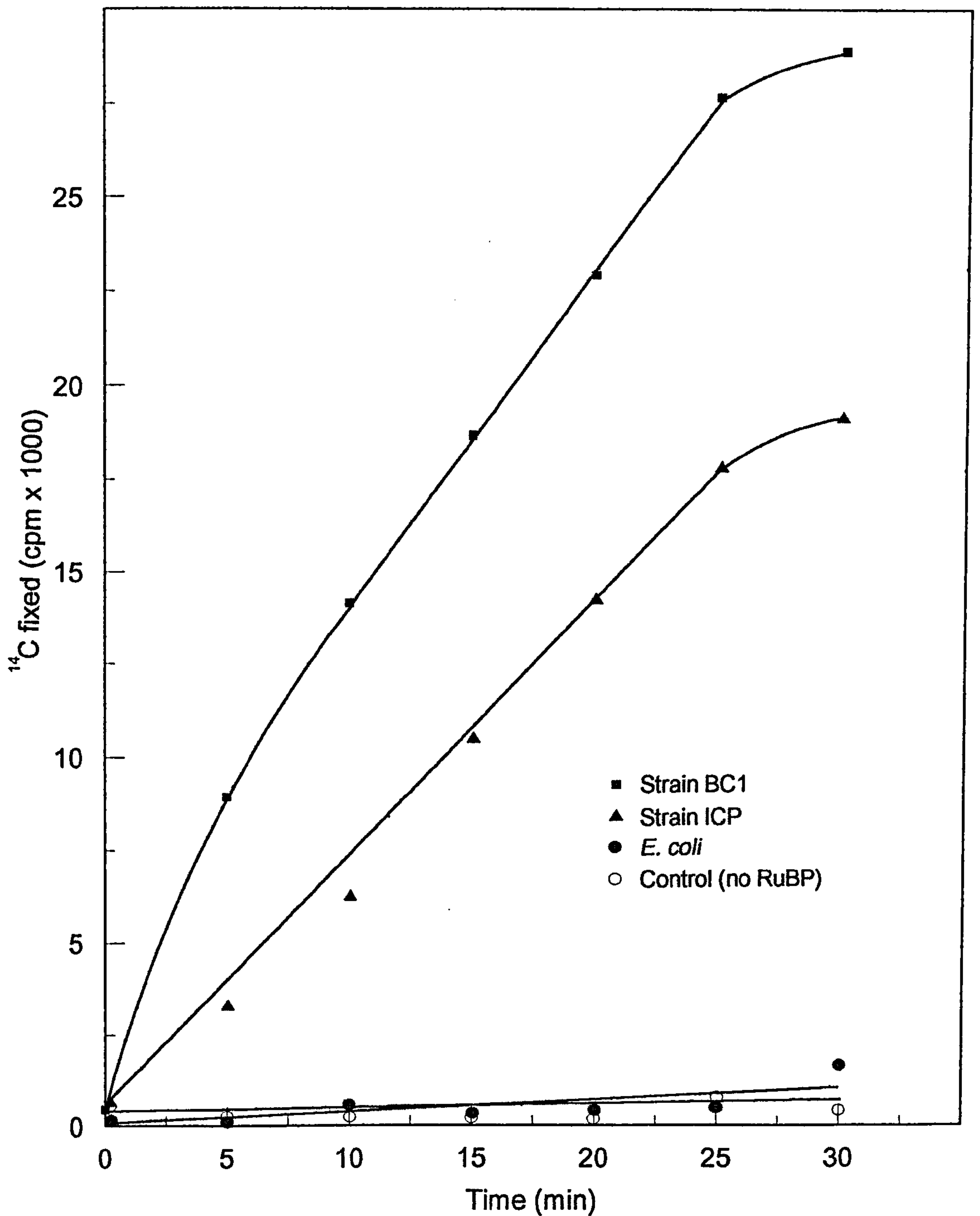


Figure 5.9: CO₂ fixed via RuBisCO during a permeabilised whole cell assay. Moderate thermophile strains BC1 and ICP were grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate, under 1% CO₂ (v/v) in air. *E. coli* was included as a control for non-RuBisCO mediated label retention. Experiments were initiated by the addition of 2 mM ribulose-1,5-bisphosphate (RuBP). Water was added instead of RuBP in the no-RuBP control.

5.3.2: Analysis of cell protein profiles of strain ICP under different CO₂ concentrations.

RuBisCO, from whatever source, generally comprises large (about 56 kDa), and small (about 15 kDa) subunits (McFadden and Majumdar, 1984), although some forms lack small subunits (Hartman *et al.*, 1984). Cells of strain ICP were subjected to SDS-PAGE analysis in an attempt to identify a protein band corresponding to the large subunit (LSU) of RuBisCO.

Anti- *T. ferrooxidans* RuBisCO large subunit (LSU) antibodies, supplied by Dr. J.C. Murrell, University of Warwick, were used to probe a western blot of denatured total cell protein samples of strain ICP. Protein samples from both chemolithoheterotrophically and autotrophically grown cells (under enhanced and limited CO₂ conditions) were probed.

The antibody clearly cross-reacted with a band in all samples at a position corresponding to approximately 56 kDa (Figure 5.10). In addition a fainter band was seen at 45 kDa. This fainter band was probably a product of the RuBisCO LSU breakdown. A much stronger cross-reaction occurred with *T. ferrooxidans* than with strain ICP, and additional bands were also visible in the *T. ferrooxidans* track. The RuBisCO from *T. ferrooxidans* has been characterised as a typical form I hexadecameric type (Holuigue *et al.*, 1987). The strong cross-reaction with strain ICP suggests that this isolate also contains the typical form I enzyme, with form I and form II enzymes differing immunologically (English *et al.*, 1992).

A clear cross-reaction was seen even in chemolithoheterotrophically grown cells, indicating that RuBisCO synthesis is not completely repressed during growth on yeast extract. It has been shown previously that strains BC1 and ALV show preferentially mixotrophic metabolism, and during mixotrophic growth on glucose still obtain at least 20% of their total carbon from CO₂ fixation (Wood and Kelly, 1989). Strain ICP probably has a similar metabolic preference, resulting in RuBisCO activity even in the presence of yeast extract.

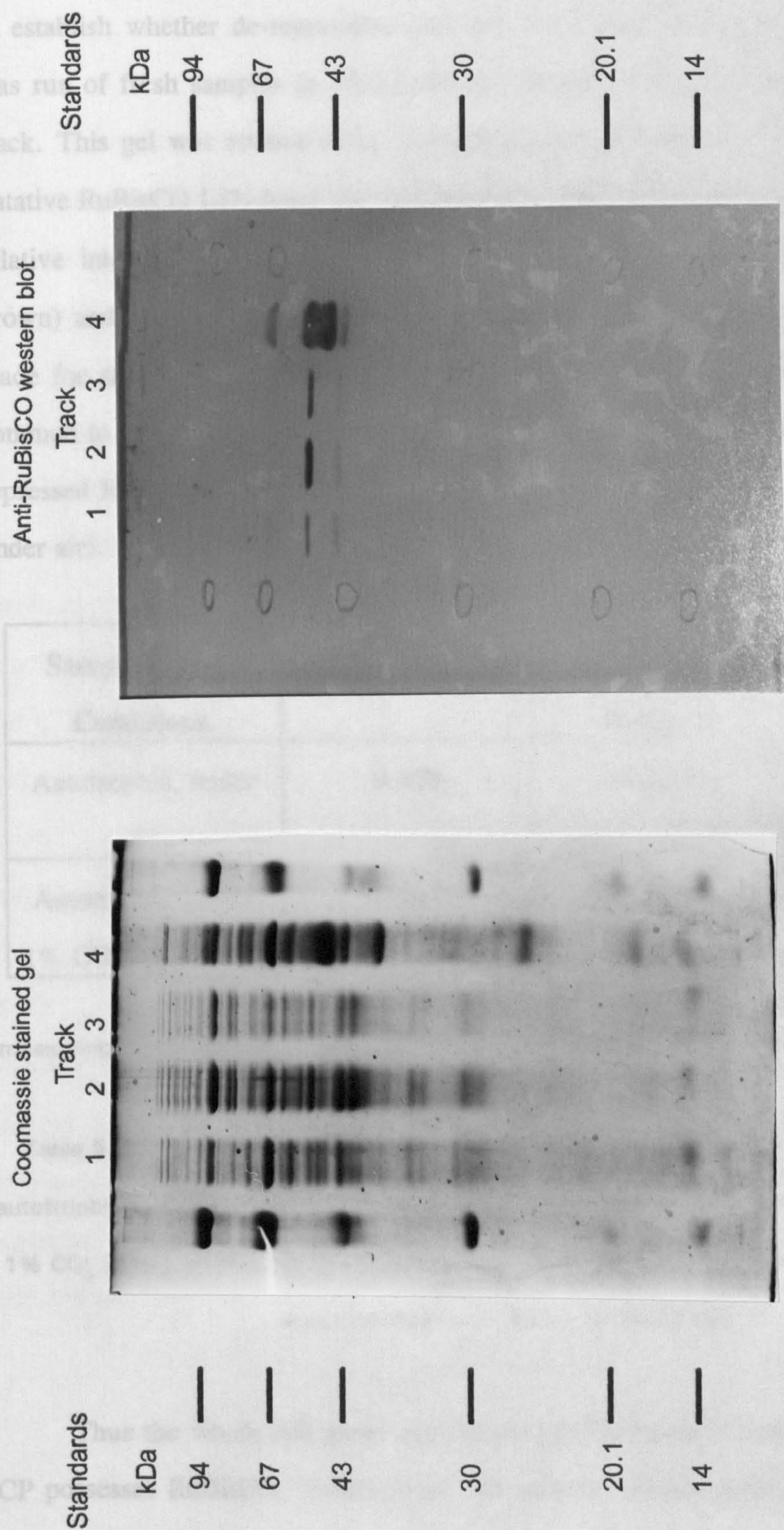


Figure 5.10: 10% (w/v) SDS-PAGE, followed by an anti-*Thiobacillus ferrooxidans* RuBisCO western blot of soluble protein extracted from strain ICP, and *T. ferrooxidans*. Strain ICP was grown chemolithoheterotrophically on 50 mM ferrous iron supplemented with 0.02% (w/v) yeast extract (track 1), autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under air (track 2), and autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under 1% (v/v) CO₂ in air (track 3). *T. ferrooxidans* was grown autotrophically on 50 mM ferrous iron under air (track 4).

RuBisCO synthesis was de-repressed 4-5 fold during autotrophic growth of *Thiobacillus* A2 under CO₂ limitation (Smith *et al.*, 1980). Similar de-repression occurs with *T. ferrooxidans* and moderate thermophile strain BC1 (MacLean, 1993). In order to establish whether de-repression occurred with strain ICP, a second denaturing gel was run of fresh samples in which similar amounts of proteins were loaded into each track. This gel was stained using Coomassie blue and analysed by densitometry. The putative RuBisCO LSU band was identified at a molecular weight of 56-57 kDa, and the relative intensity of this band was measured in the samples from CO₂ limited (air grown) and CO₂ enhanced (1% CO₂ (v/v) in air) cells (Table 5.1). A correction was made for small differences in protein loading by comparing the peak height of bands common to both tracks that were unaffected by differing CO₂ limitations. Strain ICP de-repressed RuBisCO synthesis by a factor of 2-3 during CO₂ limiting conditions (growth under air).

Sample Growth Conditions.	Peak Height.	Corrected Peak Height. ^a	De-repression Factor.
Autotrophic, under air.	9.828	8.024	1
Autotrophic, under 1% CO ₂ (v/v) in air.	3.464	3.464	2.3

^a Adjustments were made to the relative amounts of protein in each track by measuring the intensity of protein bands that were common to both tracks, but unaffected by differing CO₂ levels.

Table 5.1: Densitometry analysis of putative RuBisCO LSU band from strain ICP grown autotrophically on 50 mM ferrous iron supplemented with 0.5 mM tetrathionate under air or 1% CO₂ (v/v) in air. Protein samples were subjected to SDS-PAGE, the RuBisCO LSU band was identified as a protein of 56-57 kDa.

Thus the whole cell assay and protein profile analysis both indicated that strain ICP possesses RuBisCO. Furthermore, the specific enzyme activity, and de-repression

levels during CO₂ limited growth are of a sufficient level to sustain growth solely via CO₂ fixation.

5.3.3: PCR amplification of a RuBisCO LSU gene fragment from two moderately thermophilic iron-oxidising bacteria.

DNA fragments of 390 bp, encoding part of the RuBisCO LSU, were amplified from the genomic DNA of moderate thermophile strains ICP and NAL and sequenced. This 390 bp region is the most highly conserved portion of the RuBisCO LSU gene (Miziorko and Lorimer, 1983), and has been used previously for the comparison of RuBisCO of *T. ferrooxidans* and a moderate thermophile, NMW-6 (Holden and Brown, 1993). The PCR primers used were based on those used by Holden (1993) with modifications to reduce their large size (40-mer and 33-mer), and to increase the relatively low annealing temperature used by Holden (37 °C). Restriction enzyme sites were not required for cloning of PCR fragments using the TA cloning kit, reducing the length of both primers. The first primer used was reduced to encode only 6 amino acids, Thr-65 to Trp-70. This reduction in length gave the single codon choice for tryptophan at the 3' end of the primer, ensuring primer to template homology at this position. The second primer was also reduced in size to encode only 6 amino acids (Lys 200 to Asn 206), whilst still including the CO₂ activation site of RuBisCO.

Optimal PCR product formation was found to occur at an annealing temperature of 50 °C, with increases in annealing temperature up to 60 °C resulting in reduction or complete loss of product. Strains BC1, NAL, TH3, ICH, and ICP all gave a clear product of about 400 bp (Figure 5.11). In addition, both strains ICP and TH3 gave a second smaller product (about 150 bp). This second product in these two closely related organisms is another example of a clear difference between this moderate thermophile type and the BC1 and NAL types. The desired PCR product from strains ICP and NAL

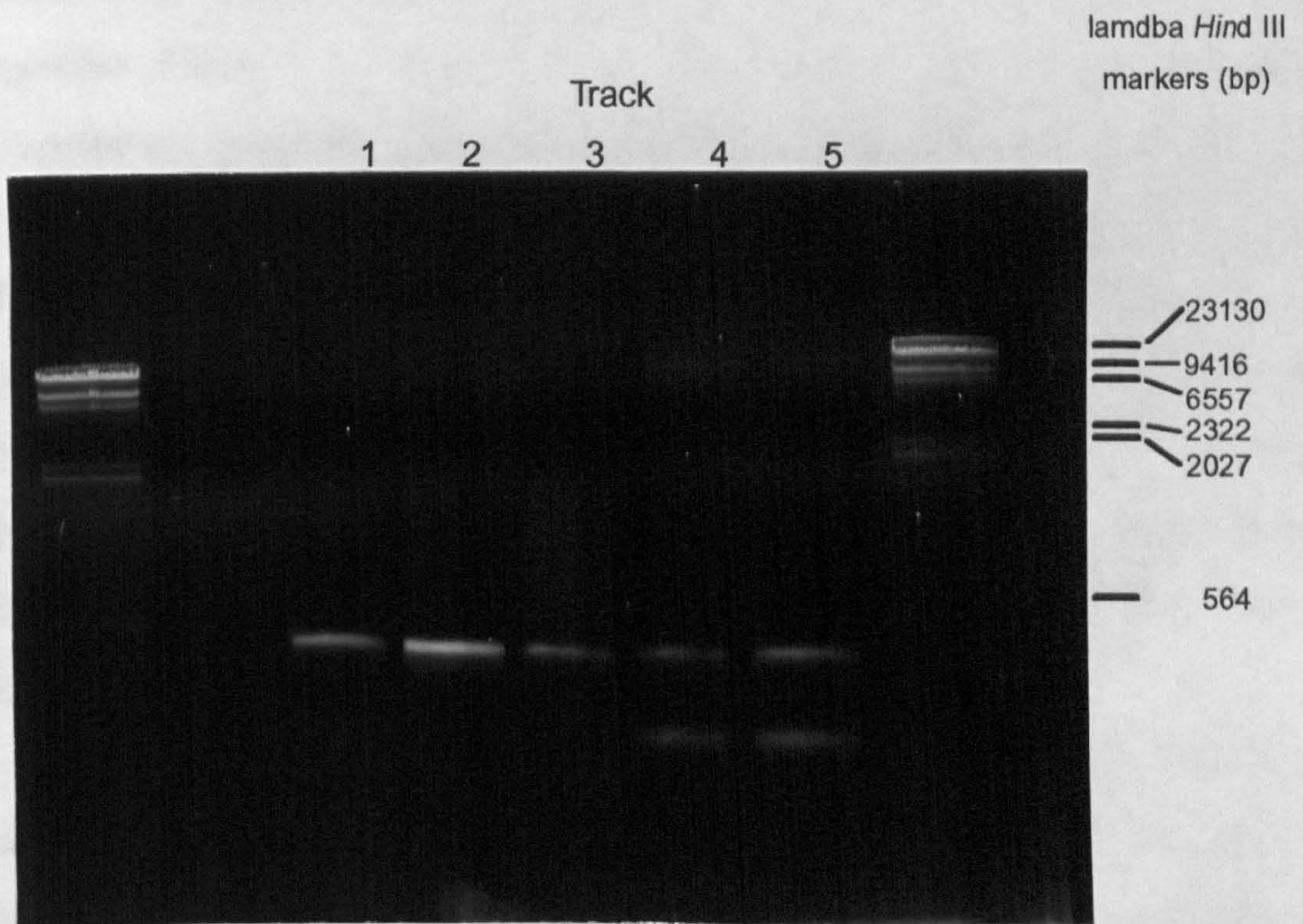


Figure 5.11: 1% (w/v) agarose minigel of PCR mix, after 30 cycles with genomic DNA as template and RuBisCO LSU primers (encoding Thr 65 to Trp 70, and Lys 200 to Asn 206). Genomic template DNA from strains BC1 (track 1), NAL (track 2), ICH (track 3), TH3 (track 4), and ICP (track 5) was used.

was purified from the PCR mix using the QIAEX gel extraction kit, cloned using the TA cloning kit, and sequenced using an automated sequencer.

The nucleotide and deduced amino acid sequences for strains NAL and ICP are shown in figure 5.12. The sequence from strain NAL encoded 129 amino acids whilst that from strain ICP encoded 130 amino acids. The amino acid sequences were compared to visually aligned sequences of the RuBisCO LSU gene from other organisms (Figure 5.13). Sequences from the following organisms were used in the comparisons: moderate thermophile NMW-6 (Holden and Brown, 1993), *T. ferrooxidans* (Kusano *et al.*, 1991a), *Alcaligenes eutrophus* (Andersen and Caton, 1987), *Chromatium vinosum* (Viale *et al.*, 1989), *Anacystis nidulans* (Shinozaki *et al.*, 1983), *Spinacea oleracea* (Zurawski *et al.*, 1981), and *Rhodospirillum rubrum* (Nargang *et al.*, 1984). The sequences from both strains NAL and ICP showed lowest nucleotide and amino acid homology to the L₂ type RuBisCO of the photosynthetic bacterium *R. rubrum* (Table 5.2), suggesting that the RuBisCOs from these two moderate thermophiles are of the typical L₈S₈ form.

For the sequence from strain NAL, the nucleotide homologies exhibited the commonly observed pattern of 2 > 1 > 3 for the three codon positions. The nucleotide sequence of strain NAL had greatest homology to the sequence of moderate thermophile NMW-6 (70.8%). Even greater homology was seen between the amino acid sequences of these two moderate thermophiles (82.2%). The lower nucleotide homology compared to amino acid homology is probably a result of the use of a high number of synonymous codons, as opposed to identical codons in these two organisms, with greatest variation in the third codon position (Table 5.2a).

a: strain NAL

210

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Thr Asp Arg Leu Thr Ala His Glu His Tyr Gln Ala Lys Ala Tyr Arg Val Val Pro
ACT GAC CGA CTG ACG GCT CAC GAG CAT TAC CAA GCG AAA GCG TAT CGC GTG GTT CCG 266

Val Pro Gly Thr Asp      Gln Tyr Ile Ala Tyr Ile Ala Tyr Asp Leu Asp Leu Phe
GTT CCC GGC ACC GAT      CAA TAT ATC GCC TAT ATC GCG TAC GAC CTG GAC CTT TTC 323

Glu Glu Gly Ser Ile Ala Asn Leu Thr Pro Ser Ile Ile Gly Asn Val Phe Gly Phe
GAA GAA GGA TCA ATC GCT AAC TTG ACG CCC TCC ATC ATT GGA AAC GTC TTC GGG TTC 380

Lys Ala Leu Lys Ser Leu Arg Leu Glu Asp Met Arg Ile Pro Pro His Tyr Val Lys
AAA GCG TTG AAG AGC CTC CGG CTG GAA GAC ATG CGA ATT CCT CCG CAC TAT GTC AAG 437

Thr Phe Gln Gly Pro Ala His Gly Ile Val Met Glu Arg Glu Tyr Leu Asn Lys Tyr
ACC TTC CAA GGA CCG GCT CAC GGG ATC GTC ATG GAG CGG GAG TAC TTG AAC AAA TAC 494

Gly Arg Pro Leu Leu Gly Ala Thr Val Lys Pro Lys Leu Gly Leu Ser Ala Arg Asn
GGA CGG CCC TTA TTG GGC GCG ACG GTC AAG CCC AAA TTG GGG TTA TCC GCC CGT AAC 551

Tyr Ala Arg Val Val Tyr Glu Ala Leu Arg Gly Gly Leu Asp Phe Thr
TAC GCA CGG GTC GTC TAT GAA GCC TTG CGC GGC GGA TTA GAC TTC ACC 599
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b: strain ICP

210

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Thr Asp Leu Leu Thr Asp Leu Asp Tyr Tyr Lys Gly Arg Ala Tyr Arg Ile Glu Asp
ACC GAC CTG CTC ACG GAC CTC GAT TAC TAC AAG GGC CGC GCC TAC CGG ATC GAG GAC 266

Val Pro Gly Asp Asp Thr Ala Phe Tyr Ala Phe Val Ala Tyr Pro Ile Asp Leu Phe
GTG CCC GGG GAC GAC ACG GCG TTC TAC GCG TTC GTC GCC TAC CCG ATC GAC CTC TTC 323

Glu Glu Gly Ser Val Val Asn Val Phe Thr Ser Leu Val Gly Asn Val Phe Gly Phe
GAA GAG GGC TCG GTC GTC AAC GTC TTC ACA TCG CTG GTC GGG AAC GTG TTC GGC TTC 380

Lys Ala Val Arg Ser Leu Pro Leu Glu Asp Val Arg Phe Pro Leu Ala Phe Val Asn
AAG GCG GTG CGA TCG CTG CCC CTC GAG GAC GTG CGC TTC CCC TTG GCC TTC GTC AAC 437

Thr Cys Asn Gly Pro Pro His Gly Ile Gln Val Glu Arg Asp Lys Met Asn Lys Tyr
ACC TGC AAC GGG CCA CCC CAC GGG ATC CAG GTC GAG CGC GAC AAG ATG AAC AAG TAC 494

Gly Arg Pro Leu Leu Gly Cys Thr Ile Lys Pro Lys Leu Gly Leu Ser Ala Lys Asn
GGC CGG CCG CTG CTC GGC TGC ACC ATC AAG CCG AAG CTC GGC CTG TCG GCC AAG AAC 551

Tyr Gly Arg Ala Val Tyr Glu Val Leu Arg Gly Gly Leu Asp Phe Thr
TAC GGG CGT GCC GTC TAC GAA GTG CTG CGC GGT GGT CTC GAC TTT ACC 599
```

Figure 5.12: The nucleotide and deduced amino acid sequences of RuBisCO LSU gene fragments

(residues 70-200, spinach numbering) amplified from genomic DNA from strains NAL (a), and ICP (b).

Numbering refers to the base position in the spinach gene.

	70	90	110	
ICP	TDLLTDLDYY	KGRAYRIEDV	PGDDTAFYAF	VAYPIDLFEE
NAL	..R..AHEH.	QAK...VVP.	..T.-QYI.Y	I..DL.....
NMW	R..AY.N.	QAK...VDQI	..T.-QYF.Y	I..S.....
TfM...C....
Ae	..R..AC.M.	RAK...VDP.	.NNPEQ.FCY	...DLS....
CvA....C....	I.....
AnM.R.	..KC.H..P.	Q.EENSIFY..	I...L.....
So	..G..N..R.	...C.H..P.	A.EENQYICY	...L.....
			↓	↓ ↓
Rr	T---...F.R	.VDALVYEVD	EARELTKI.Y	..LFDRNITD
				.KAMAS..LT
				M..NQ.MGD.
	130	150	170	190
	RSLPLEDVRF	PLAFVNTCNG	PPHGIQVERD	KMNKYGRPLL
	GCTIKPKLGL	SAKNYGRAVY	EVLRGGLDFT	
K..R...	M.I	.PHY.K.FQ.	.A...VM..E	YL.....
.A.V.....	..R..A.V..	.A.....	..R....VA.	.A.....
KA.R...	M.I	..HYIK.FQ.	.A...V...E	YLD.....
.A.R.....	...Y.K....M.C.....
KAAR...	M..	.V.Y.K.FA.	.ST..I...E	RLD.F.....
.A.T.....	.GR....V..	.G.K....M		
.A.R.....	.I.Y.M....	...A.....	I.....M.
...R...I..	.V.L.K.FQ.LL.....	M.
.A.R...L.I	.V.Y.K.FQ.L.....C.....
			↓ ↓	
EYAKMH.FYV	.E.YRALFD.	.SVN.SALW.	K...V...VV	.TI..P....
				RP.PFAE.CH
				.AFWL....I

Figure 5.13: Comparison of the deduced amino acid sequences of RuBisCO LSU gene fragments from moderate thermophile strains ICP and NAL with those of other organisms. Visually aligned sequences from the LSU gene of the moderate thermophile NMW-6 (NMW), *T. ferrooxidans* (Tf), *Alcaligenes eutrophus* (Ae), *C. vinosum* (Cv), *Anacystis nidulans* (An), higher plant *Spinacea oleracea*, and photosynthetic bacterium *R. rubrum* are shown. Dots indicate the presence of the same residue at that position. Dashes indicate deletions. Arrows indicate positions where insertions in the *R. rubrum* sequences have been omitted to allow alignment. Numbering refers to residue positions in the spinach enzyme.

a: strain NAL

Species	Sequence homology (%):			
	Amino Acids Identical	Codons		
		Identical	Synonymous	Replacement
NMW6	82.2	31.1	51.1	17.8
<i>T. ferrooxidans</i>	62.8	26.4	36.4	37.2
<i>A. eutrophus</i>	72.8	26.4	46.4	27.2
<i>C. vinosum</i>	60.5	25.6	34.9	39.5
<i>A. nidulans</i>	65.9	29.5	36.4	34.1
<i>S. oleracea</i>	69.0	31.0	38.0	31.0
<i>R. rubrum</i>	24.8	13.2	11.6	75.2

Species	Nucleotides Sequence Homology (%):			
	Total	Codon position		
		1	2	3
NMW6	70.8	82.9	91.5	38.0
<i>T. ferrooxidans</i>	61.8	66.7	79.8	39.5
<i>A. eutrophus</i>	65.6	73.6	81.4	41.8
<i>C. vinosum</i>	60.9	70.0	76.7	38.8
<i>A. nidulans</i>	63.3	72.1	79.8	38.0
<i>S. oleracea</i>	64.3	72.9	82.9	39.5
<i>R. rubrum</i>	42.4	42.1	47.9	40.5

b: strain ICP

Species	Sequence homology (%):			
	Amino Acids Identical	Codons		
		Identical	Synonymous	Replacement
Strain NAL	62.0	25.6	36.4	38.0
NMW6	62.3	18.5	43.8	37.7
<i>T. ferrooxidans</i>	93.8	50.8	43.0	6.2
<i>A. eutrophus</i>	56.2	33.1	23.1	43.8
<i>C. vinosum</i>	90.8	63.1	27.7	9.2
<i>A. nidulans</i>	77	48.5	28.5	23
<i>S. oleracea</i>	76.2	19.2	57.0	23.8
<i>R. rubrum</i>	31.1	18.0	13.1	68.9

Species	Nucleotides Sequence Homology (%):			
	Total	Codon position		
		1	2	3
Strain NAL	61.5	69.8	78.3	36.4
NMW6	60.9	71.7	80.3	29.1
<i>T. ferrooxidans</i>	81.8	93.1	96.2	54.6
<i>A. eutrophus</i>	66.4	70.0	71.5	57.7
<i>C. vinosum</i>	85.1	94.6	93.8	65.4
<i>A. nidulans</i>	76.2	83.8	89.2	56.9
<i>S. oleracea</i>	64.1	79.2	87.7	26.2
<i>R. rubrum</i>	45.4	48.4	45.9	41.8

Table 5.2: Comparison of the nucleotide and deduced amino acid sequence homologies of RuBisCO LSU gene fragments from moderate thermophile strains NAL (a) and ICP (b) with those of other organisms.

Both the sequences from strains NAL and NMW-6 differed from all the other hexadecameric RuBisCO sequences in only encoding for 129 amino acids within the region amplified (with a deletion at position 94, spinach numbering). 35 substitutions occurred in the NAL amino acid sequence from that of the spinach sequence, of which 23 were identical to substitutions in NMW-6, a further indication of the high RuBisCO homology between these two organisms. The sequence from the chemolithotroph *Alcaligenes eutrophus* had the next greatest homology to strain NAL; 72.8% for amino acids and 65.6% for nucleotides. These values are almost identical to those obtained in a comparison of NMW-6 and *Alcaligenes eutrophus* (Holden and Brown, 1993). The high homology values obtained between strain NAL and NMW-6 suggests that these two organisms are closely related *Sulfobacillus*-like bacteria.

The nucleotide homology values of the sequence from strain ICP compared to other hexadecameric RuBisCO sequences ranged from 60.9% to 85.1%, with the least homology to moderate thermophiles NMW-6 and NAL. This is another indication (see DNA:DNA hybridisation studies, and analysis of G+C content, chapter 3) of the taxonomic difference between these two types of moderate thermophiles. The sequence from strain ICP had greatest nucleotide homology to the sequence from the photosynthetic bacterium *C. vinosum* (85.1%), and *T. ferrooxidans* (81.8%). With the exception of *R. rubrum*, and *C. vinosum* the homologies exhibited the commonly observed pattern of $2 > 1 > 3$ for the three codon positions.

The amino acid homologies of the deduced sequence from strain ICP varied from 56.2% to 93.8%, with greatest homology to *T. ferrooxidans*. Thirty one amino acid substitutions occurred in the ICP sequence from that of spinach, and of these 25 were identical to substitutions in the *T. ferrooxidans* sequence. Eight differences occurred in the amino acid sequences of strain ICP and *T. ferrooxidans*, of which only two were in the most conserved region of the gene (positions 161 to 192, spinach numbering). This is a very high homology even for such a highly conserved gene, and suggests similar evolution of the RuBisCO genes of these two iron-oxidizing autotrophs.

Investigation of the codon usage of the RuBisCO LSU gene fragment of strains ICP and NAL indicated a strong bias towards high G+C containing codons in strain ICP (Table 5.3). All arginine codons used had C instead of A at position 1, and all but one leucine codon had C instead of T at position 1. This G+C bias was more evident in the variable third codon position. A similar bias has been observed in the RuBisCO LSU gene of *Alcaligenes eutrophus* (Andersen and Caton, 1987), and is probably a reflection of the high G+C content of these two organisms (68 and 66 mol% for strain ICP and *Alcaligenes eutrophus* respectively). Strain NAL had a much less biased codon usage, reflecting its lower overall G+C content (55 mol%).

a b				a b				a b				a b			
Phe	TTT		1	Ser	TCT			Tyr	TAT	5		Cys	TGT		
Phe	TTC	5	8	Ser	TCC	2		Tyr	TAC	5	8	Cys	TGC		2
Leu	TTA	3		Ser	TCA	1		End	TAA			End	TGA		
Leu	TTG	6	1	Ser	TCG		4	End	TAG			Trp	TGG		
Leu	CTT	1		Pro	CCT	1		His	CAT	1		Arg	CGT	1	1
Leu	CTC	1	7	Pro	CCC	4	4	His	CAC	3	1	Arg	CGC	2	4
Leu	CTA			Pro	CCA		1	Gln	CAA	3		Arg	CGA	2	1
Leu	CTG	2	6	Pro	CCG	3	3	Gln	CAG		1	Arg	CGG	4	2
Ile	ATT	2		Thr	ACT	1		Asn	AAT			Ser	AGT		
Ile	ATC	5	4	Thr	ACC	3	4	Asn	AAC	4	6	Ser	AGC	1	
Ile	ATA			Thr	ACA		1	Lys	AAA	4		Arg	AGA		
Met	ATG	2	1	Thr	ACG	3	2	Lys	AAG	3	7	Arg	AGG		
Val	GTT	2		Ala	GCT	4		Asp	GAT	1	1	Gly	GGT		2
Val	GTC	6	8	Ala	GCC	3	5	Asp	GAC	5	9	Gly	GGC	3	6
Val	GTA			Ala	GCA	1		Glu	GAA	4	2	Gly	GGA	5	
Val	GTG	1	5	Ala	GCG	5	3	Glu	GAG	3	4	Gly	GGG	3	5

Table 5.3: Comparison of the codon usage in a fragment of the RuBisCO LSU gene (residues 70-200) from moderate thermophiles NAL (a), and ICP (b).

5.4: Discussion.

During autotrophic growth under air, the moderately thermophilic mixed culture ICC was found to induce a high affinity CO₂ uptake system (Figure 5.3). A similar high affinity uptake system has been demonstrated in *T. neapolitanus* (Holthuijzen *et al.*, 1987), and this organism is capable of autotrophic growth under air. In *T. versutus* such a high affinity uptake system was found to be lacking and consequently the organism grew poorly autotrophically under air (Karagouni and Kelly, 1989). This clearly suggests that a high affinity CO₂ uptake system is essential for good autotrophic growth under air.

The low CO₂ uptake rate seen with culture ICC under air levels of CO₂ (30 nmol CO₂/min/mg protein), a level that was believed to be insufficient to support growth of moderate thermophile strain BC1 (MacLean, 1993), was a result of only one of the two organisms within this mixed culture contributing to the high affinity uptake system. The second isolate (strain ICH) possessed only a low affinity CO₂ uptake system, but is believed to be essential for good autotrophic growth of the mixed culture under air, because of the apparent whole cell affinities of strain ICP to ferrous and ferric iron (see section 4.4.3).

The possession of a high affinity CO₂ uptake system by strain ICP, therefore, appears to be the key factor in the growth of the mixed culture under air. Strain ICH does not possess such a system and probably obtains additional fixed carbon from strain ICP.

The presence of RuBisCO, the key enzyme of the Calvin cycle, within an organism can be used as a direct indication of the operation of the Calvin cycle (Tabita, 1988). Whole cell RuBisCO assays confirmed the presence of RuBisCO within strain ICP at a level sufficient for efficient CO₂ fixation (Wood and Kelly, 1985).

Molecular analysis of the RuBisCO enzyme from strain ICP indicated that it was of the typical hexadecameric (L₈S₈) form. This was evident from both western blotting of total cellular proteins with anti-RuBisCO antibodies (Fig. 5.10), and by the analysis

of a fragment of the RuBisCO LSU gene from strains ICP and NAL (Fig. 5.13, and Table 5.2).

The RuBisCO LSU gene fragment from strain ICP was found to be highly homologous (93.8% amino acid homology) to that of *T. ferrooxidans*, a Gram-negative, mesophilic iron-oxidizing autotroph. This high homology raises the question; why does the RuBisCO gene sequence of strain ICP show greater homology to the gene from *T. ferrooxidans*, than that from other moderately thermophilic iron-oxidizing bacteria? Three possible explanations for this high homology are:

1. Strain ICP and *T. ferrooxidans* share a common ancestry from which they diverged relatively recently in evolutionary terms.
2. Convergent evolution of the RuBisCO genes has occurred in strain ICP and *T. ferrooxidans*.
3. Lateral transfer of the RuBisCO genes has occurred between strain ICP and *T. ferrooxidans*.

Despite strain ICP and *T. ferrooxidans* having similar metabolic requirements, 16S rRNA sequence analysis indicates that Strain TH3 (an ICP type organism) and *T. ferrooxidans* are distantly related organisms (Lane *et al.*, 1992). This would suggest recent divergence from a common ancestry is unlikely.

Convergent evolution of RuBisCO in these two organisms would probably have resulted in several short areas of the gene having high homology (i.e. those specifically involved in conformational changes of the active site, *etc.*), rather than the whole of a 390 bp region.

Lateral gene transfer of the mercuric reductase gene is believed to have occurred from *Pseudomonas* (or a similar organism) to *T. ferrooxidans* via transposon Tn501 or plasmid R100 (Inoue *et al.*, 1989). This gene had a different G+C content and different codon usage to that found in the rest of the *T. ferrooxidans* genome. No such lateral transfer has been described for RuBisCO genes, but in at least one case (with *Alcaligenes eutrophus*) two sets of RuBisCO genes occur, with one set encoded on a self-transmissible megaplasmid (Andersen and Wilke-Douglas, 1984). If a copy of the

RuBisCO genes occurred on such a plasmid in either strain ICP or *T. ferrooxidans*, then lateral gene transfer would be possible, as these two organisms are likely to share the same habitats. However, both the RuBisCO genes from *T. ferrooxidans* (Kusano, Takeshima et al, 1991), and the RuBisCO LSU gene fragment from strain ICP have G+C contents and codon usage consistent with their own genomes. Therefore, unless the codon usage and G+C content of the rest of the RuBisCO LSU gene from strain ICP differs significantly from that of the fragment sequenced here, lateral gene transfer of RuBisCO between strain ICP and *T. ferrooxidans* is unlikely.

At present the reason for high homology between the RuBisCO LSU genes of strain ICP and *T. ferrooxidans* is not clear, but completion of the sequencing of the RuBisCO large and small subunits of strain ICP might resolve this situation.

In common with *T. ferrooxidans*, the RuBisCO LSU gene fragment from strain ICP was more closely related to RuBisCO genes of photosynthetic origin, than that of the chemolithoautotroph *Alcaligenes eutrophus* (Figure 5.14). In contrast the fragments from both strain NAL and moderate thermophile NMW-6 appeared more closely related to that of *Alcaligenes eutrophus*, than sequences of photosynthetic origin (Fig. 5.14). This difference in homology between the RuBisCO LSU gene sequences of these moderate thermophiles suggests that the evolution of their RuBisCO genes diverged at an early stage. It has been suggested that the molecular evolution of RuBisCO developed from an ancient photosynthetic bacterium to the form II RuBisCO of *R. rubrum*, and then to the hexadecameric form I RuBisCO of the cyanobacteria (McFadden *et al*, 1986). From this point the molecular evolution of RuBisCO is thought to have diverged into that found in chemosynthetic bacteria, and that of algae and higher plants. Therefore, the high homology of the RuBisCO LSU gene sequence of strain ICP to RuBisCO genes of photosynthetic origin and particularly of cyanobacteria (e.g. *Anacystis nidulans*) may indicate that this moderately thermophilic iron-oxidiser is a more ancient type than the *Sulfobacillus*-type organisms.

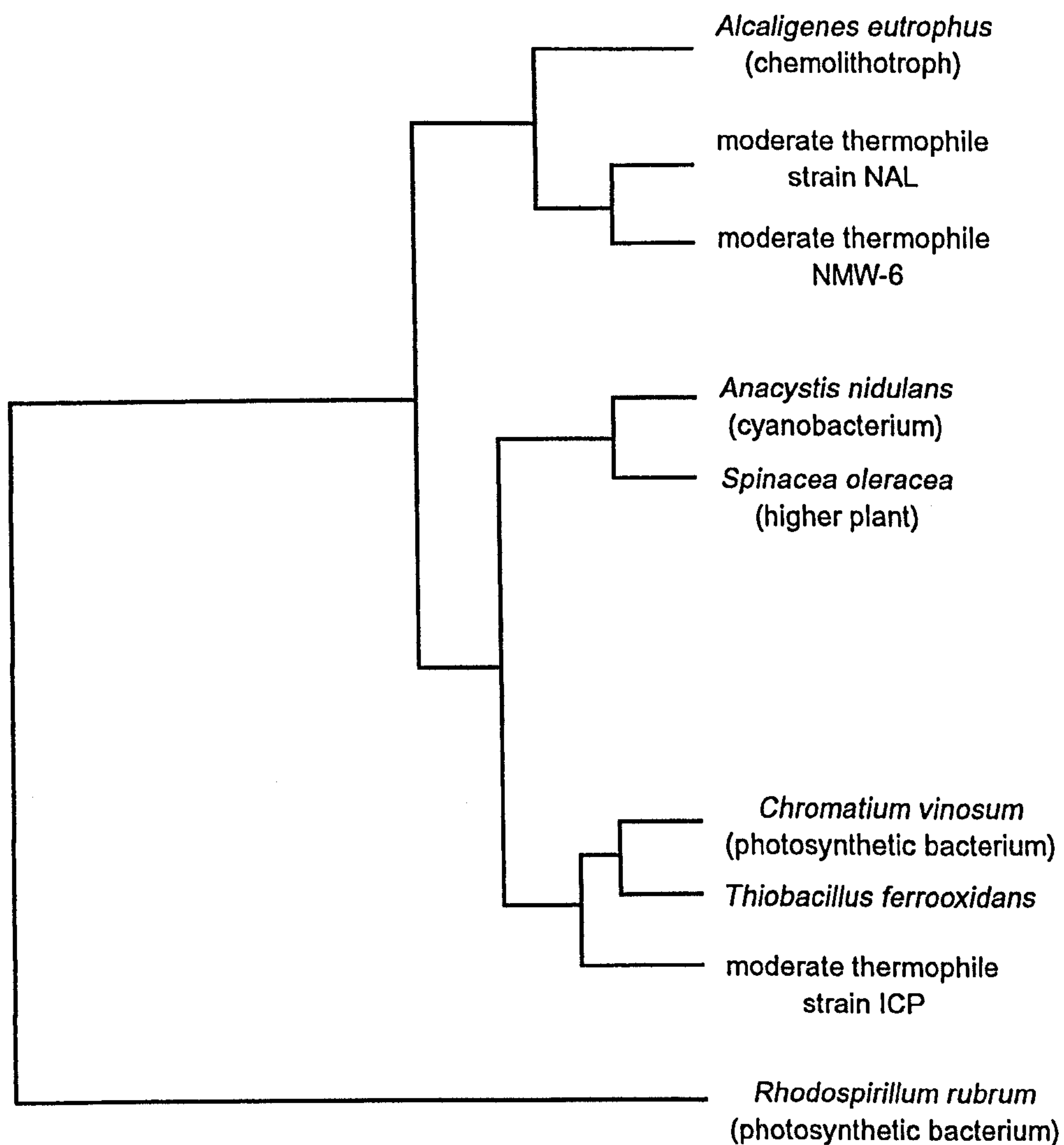


Figure 5.14: Comparison of a RuBisCO LSU gene fragment (residues 70-200, spinach numbering) from moderate thermophile strains ICP and NAL, compared with the sequences from other organisms. Deduced amino acid sequences were aligned and a tree of sequence relatedness produced using the pileup, distances and growtree functions of the GCG sequence analysis software package (Devereux et al. 1984). UPGMA and the Kimura 2-protein correction were used to produce the tree.

Chapter 6:

Mineral Leaching Studies.

6.1: Introduction.

Exhaustion of many rich metal deposits, has resulted in a need to utilise lower grade ores, and there has been increasing pressure to extract metals in an environmentally friendly and efficient manner. This has resulted in increased interest in the use of biological oxidation processes where mineral sulphides contain target metals. Natural metal leaching has been exploited since Roman times to obtain a few metals such as copper, but it has only been in the last four decades that the role micro-organisms play in this mineral dissolution has become clear (Lundgren and Silver, 1980). Microbial leaching usually involves the use of dumps or heaps of low grade ore, through which leach solution is trickled (Hutchins *et al.*, 1986). The leach solutions are then collected and the metals recovered.

A more recent development has been in the recovery of precious metals, such as gold, by the bio-oxidation of refractory ores in reactors. It is estimated that between 15-30% of the world's gold is encapsulated by minerals rendering them refractory (Barrett *et al.*, 1993). A refractory ore is simply one from which the precious metal cannot be obtained economically by conventional processing technologies (Hutchins *et al.*, 1987). The traditional method for treating these ores is by roasting, but this results in atmospheric pollution. The two main alternatives to roasting are pressure oxidation and bacterial oxidation (Barrett *et al.*, 1993). The choice between the two alternatives is usually based on economic factors, and bacterial oxidation appears to be gaining in favour.

The use of thermophilic cultures for bio-oxidation has progressed to the pilot plant scale, and there are numerous examples of their potential for improved rates of mineral dissolution (Spencer *et al.*, 1989; Carlson *et al.*, 1992; Budden and Spencer, 1993). Rarely have mesophilic and thermophilic cultures been compared directly on a range of minerals. Therefore, the aim of this work was to compare, the growth and mineral-oxidation rates of a mesophilic culture, a moderately thermophilic culture, and an extremely thermophilic culture, on a range of minerals.

The three ore concentrates used in the experiments were a Mexican pyrite and the Bogosu arsenopyrite (obtained from Shell Research Limited), and the Olympia arsenopyrite concentrate (originating from Greece). For mineralogical analysis of these concentrates see section 2.2.3. The Mexican pyrite was chosen as a very pure pyrite substrate, which has been routinely used within this laboratory to maintain stock cultures. The Bogosu arsenopyrite was included because it was a mineral of interest to Shell Research Ltd., and the Olympia concentrate was included because of its higher arsenic content.

The three cultures used in these experiments were a mesophilic mixed culture, a moderately thermophilic mixed culture, and the extreme thermophile *Sulfolobus* strain BC. The mixed cultures consisted of all the available mesophilic or moderately thermophilic organisms within the laboratory combined as a single inoculum and grown for several sub-cultures on pyrite. Each culture was grown close to its optimum growth temperature of 30, 48, and 68 °C respectively.

6.2: Leaching of the Mexican pyrite concentrate.

When 2% (w/v) Mexican pyrite was leached during growth of the three cultures, similar results were obtained with each culture (Figure 6.1). The mesophilic culture took about 100 hours before maximal solubilisation rates occurred. However, this would likely be of little consequence in any industrial application, where continuous reactors are used. The maximum iron solubilisation rate achieved by the mesophilic culture was 31.6 mg/l/h, and a total mineral dissolution of about 81% was achieved. Sulphate production followed a similar trend to that of iron, but indicated more complete solubilisation of the mineral, at about 92%. This difference between total iron release and sulphate production in solution is probably a result of iron precipitation during the experiment, resulting in the formation of hydrated iron oxides or secondary minerals, and a consequent reduction of iron in solution (Hutchins *et al.*, 1987). The pH fell from 1.94 to 1.11 during the course of the experiment.

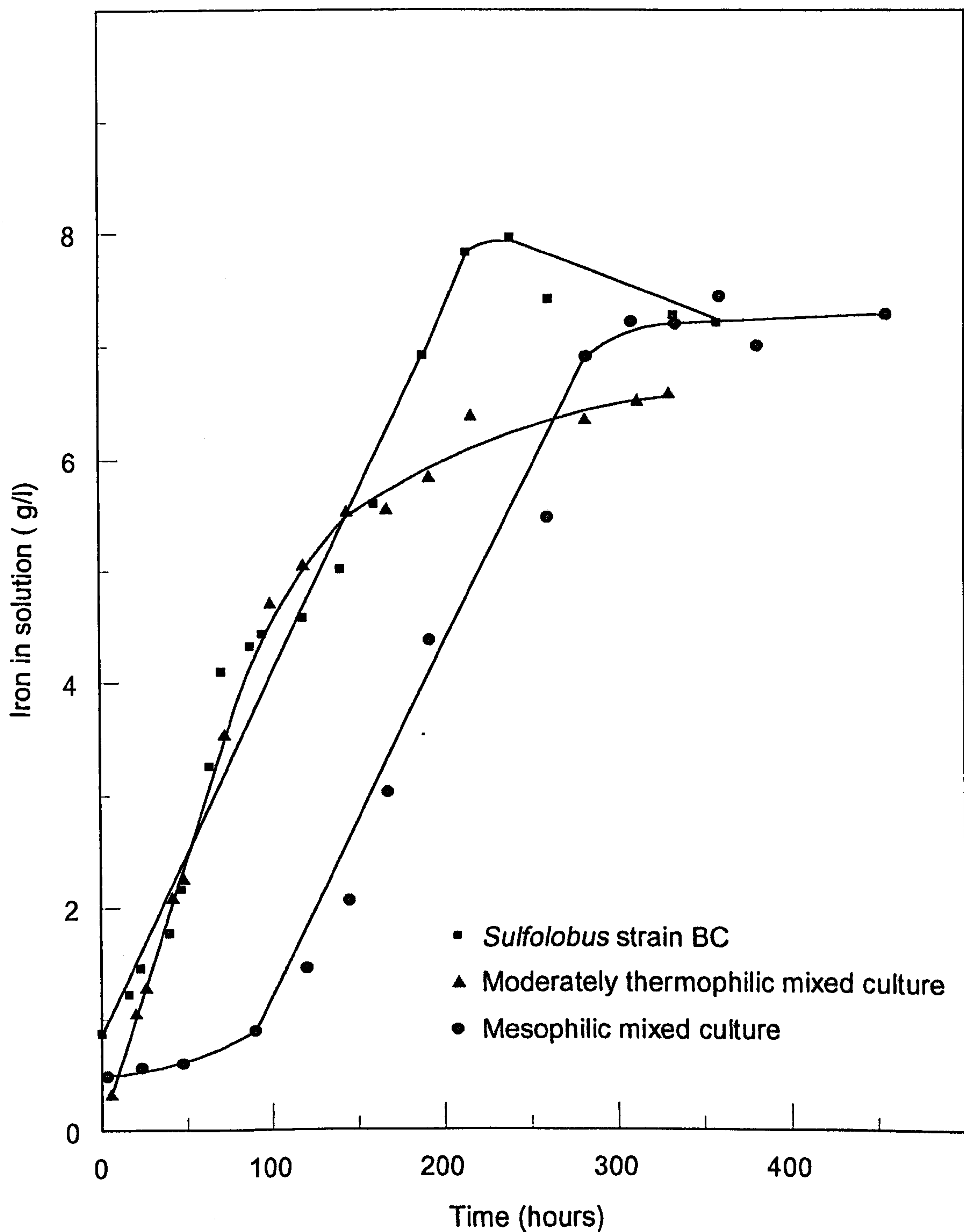


Figure 6.1: The solubilisation of 2% (w/v) Mexican pyrite concentrate during growth of three mineral-oxidizing cultures; a mesophilic mixed culture grown at 30°C, a moderately thermophilic mixed culture grown at 48°C, and the extreme thermophile *Sulfolobus* strain BC grown at 68°C. The mesophilic culture was gassed with air, whilst both the moderately thermophilic culture and *Sulfolobus* strain BC were gassed with 1% (v/v) CO₂ in air.

The moderately thermophilic culture produced a faster maximum iron solubilisation rate of 43.3 mg/l/h, but only 73 % mineral dissolution appeared to occur. The total mineral dissolution figure was again slightly higher using sulphate production figures at 80%, but was still significantly lower than that achieved by the mesophilic culture. This lower total mineral dissolution is probably caused by the final pH of 1.20 being inhibitory, as the moderately thermophilic organisms have a lower acid tolerance than the acidophilic thiobacilli (Norris *et al.*, 1986). A 70-80% mineral dissolution level may not restrict commercial application, because often only partial mineral dissolution is required to expose the desired metal for conventional treatment (Olson, 1994), but it is more likely that continuous pH control would be practised to facilitate maximum mineral oxidation.

The extreme thermophile *Sulfolobus* strain BC produced similar maximal iron solubilisation rates to the mesophilic culture (32.7 mg/l/h), but this maximal rate occurred almost immediately after inoculation, and greater mineral dissolution occurred; 82% by iron solubilisation, 98% by sulphate production. The large differences between the iron and sulphate value is probably a result of increased iron precipitation at higher temperatures. *Sulfolobus* strain BC produced the lowest final pH at 1.03.

During this experiment, a low ferrous iron level of around 50-100 mg/l was seen throughout the time course, after an initial increase to 200-500 mg/l, with the mesophilic and moderately thermophilic cultures. This indicates that the bacteria were oxidizing the available ferrous iron rapidly. The ferrous iron reached a higher maximum level during the leaching by *Sulfolobus* strain BC, rising to 1.7 g/l after 95 hours, before falling rapidly to the level seen with the other cultures of about 100 mg /l.

6.3: Leaching of the Bogosu arsenopyrite concentrate.

The Bogosu concentrate originates from a site in Africa close to the site of the Ashanti Goldfields Corporation Ltd. mine at Sansu, where the largest bio-oxidation plant in the world is operating (Nicholson and Oti-Atakorah, 1993). The batch of mineral concentrate used in these experiments had an arsenic content of almost 4% (twice that of the mineral being processed at Sansu). Figure 6.2 shows the leaching of 2% (w/v) Bogosu concentrate, during the growth of the three cultures. This mineral was leached rapidly by all three cultures, but at different rates. The rate differences were clearly temperature related with the mesophilic culture having the slowest rate of mineral dissolution and the extreme thermophile *Sulfolobus* strain BC the fastest rate.

The mesophilic culture had a shorter period prior to iron release than during growth on the Mexican pyrite, but this was still much greater than that seen with the other two cultures. The maximal rates for iron dissolution and sulphate production by the mesophilic culture were 12.5 mg/l/h and 38 mg/l/h. There was a large discrepancy in the total mineral dissolution as estimated by iron release and sulphate production, at 84% and 56% respectively. The final pH was 1.58 after an initial increase from 2.00 to 2.32. This relatively high final pH may be an indication that a greater proportion of sulphur remained in the mineral, resulting in less sulphuric acid production, or it may be caused by the release of alkaline gangue material from the mineral raising the pH. The release of alkaline material is the more likely explanation, as the pH increased immediately upon addition of mineral to the acidic media. It is not known what affect a reduced sulphur oxidation level would have on final precious metal recovery (depending on the mineralogy of the concentrate, and how the precious metal was distributed within it).

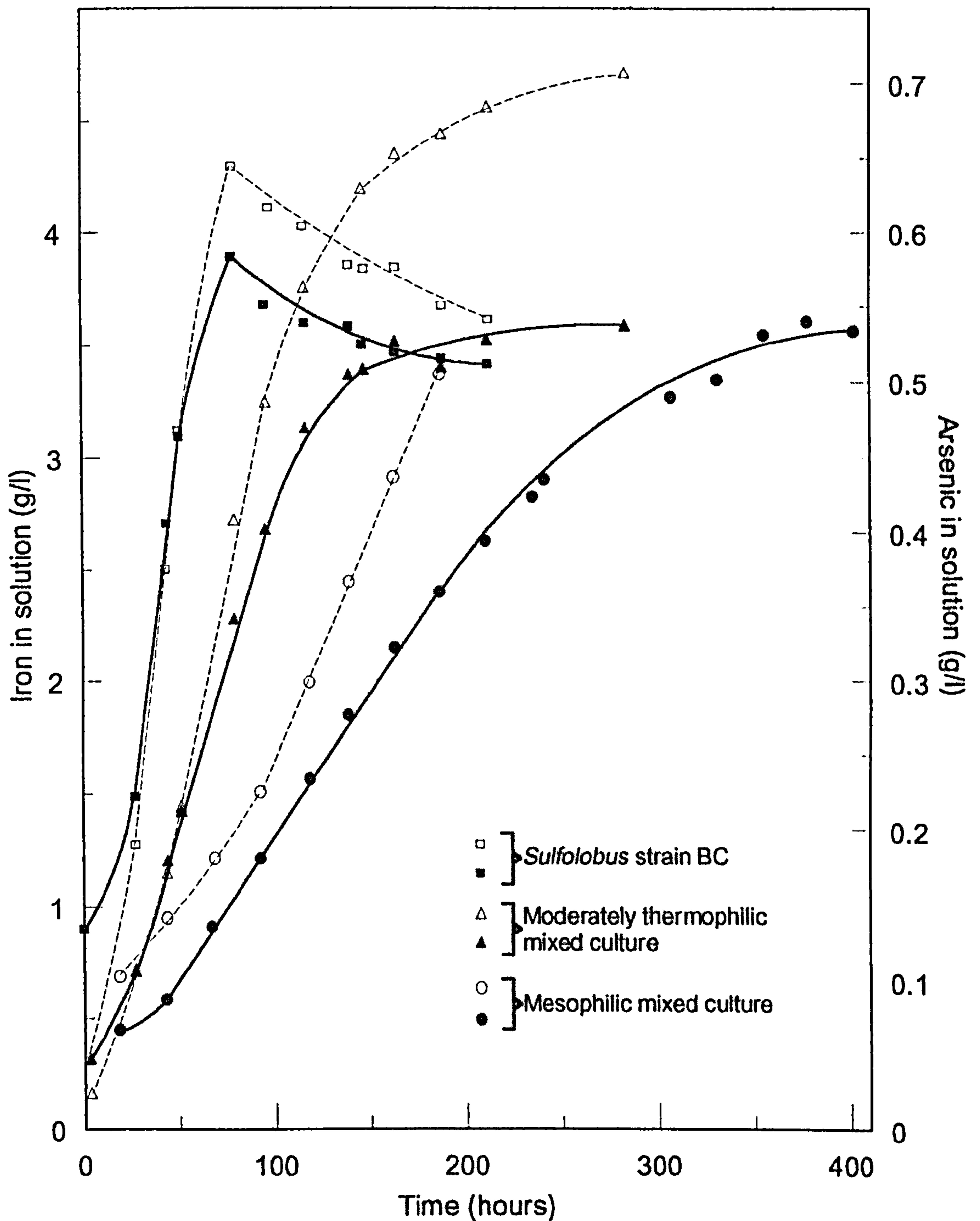


Figure 6.2: The solubilisation of 2% (w/v) Bogosu arsenopyrite concentrate during growth of three mineral-oxidizing cultures; a mesophilic mixed culture grown at 30°C, a moderately thermophilic mixed culture grown at 48°C, and the extreme thermophile *Sulfolobus* strain BC grown at 68°C. The mesophilic culture was gassed with air, the moderately thermophilic culture and *Sulfolobus* strain BC were gassed with 1% (v/v) CO₂ in air. Open symbols and dashed lines represent arsenic concentrations; closed symbols and solid lines represent iron concentrations.

The moderately thermophilic culture showed over twice the maximal mineral solubilisation rate of the mesophilic culture, at 30 mg/l/h for iron release and 75 mg/l/h for sulphate production. The total mineral dissolution value obtained from iron and sulphate solubilisation data was in close agreement, at 85% and 80% respectively. The pH was again seen to increase above 2.00 before dropping to a final value of 1.28, significantly lower than the mesophilic culture.

The extreme thermophile produced the fastest rates of solubilisation at 70 mg/l/h for iron release and 225 mg/l/h for sulphate production, over twice the rates of the moderately thermophilic culture, and approximately 6 times the rates of the mesophilic culture. Total apparent mineral dissolution was similar to that achieved with the moderately thermophilic culture at 80-85%, and the pH dropped to a similar value (pH 1.33).

The ferrous iron levels remained at low concentration in solution (30-50 mg/l), after an initial small increase (about 200 mg/l), indicating that it was rapidly oxidized by all three cultures. This contrasts to the situation with the Mexican pyrite, where much higher levels of ferrous iron in solution were seen with *Sulfolobus*.

Figure 6.2 also indicates that arsenic was released into solution in proportion to iron release. 85-100% of the total arsenic was released into solution by the end of the time course. The final arsenic values for the mesophilic culture were not obtained because of an equipment fault whilst on placement with Shell Research Ltd., but the results suggest similar values to those obtained for the other two cultures. The arsenic level of 700 mg/l was not inhibitory.

In an attempt to ascertain whether arsenic levels would become inhibitory, leaching experiments were carried out using 10% (w/v) Bogosu concentrate (Figure 6.3). Sulphuric acid was added at the start of the experiment to maintain the initial pH at 2.00-2.30, as addition of mineral otherwise resulted in a rapid rise in the pH to above 6.0. The mesophilic and moderately thermophilic cultures produced similar results to those at 2% (w/v) mineral, but the extreme thermophile *Sulfolobus* strain BC failed to grow at this higher mineral concentration. This failure to grow at 10% (w/v)

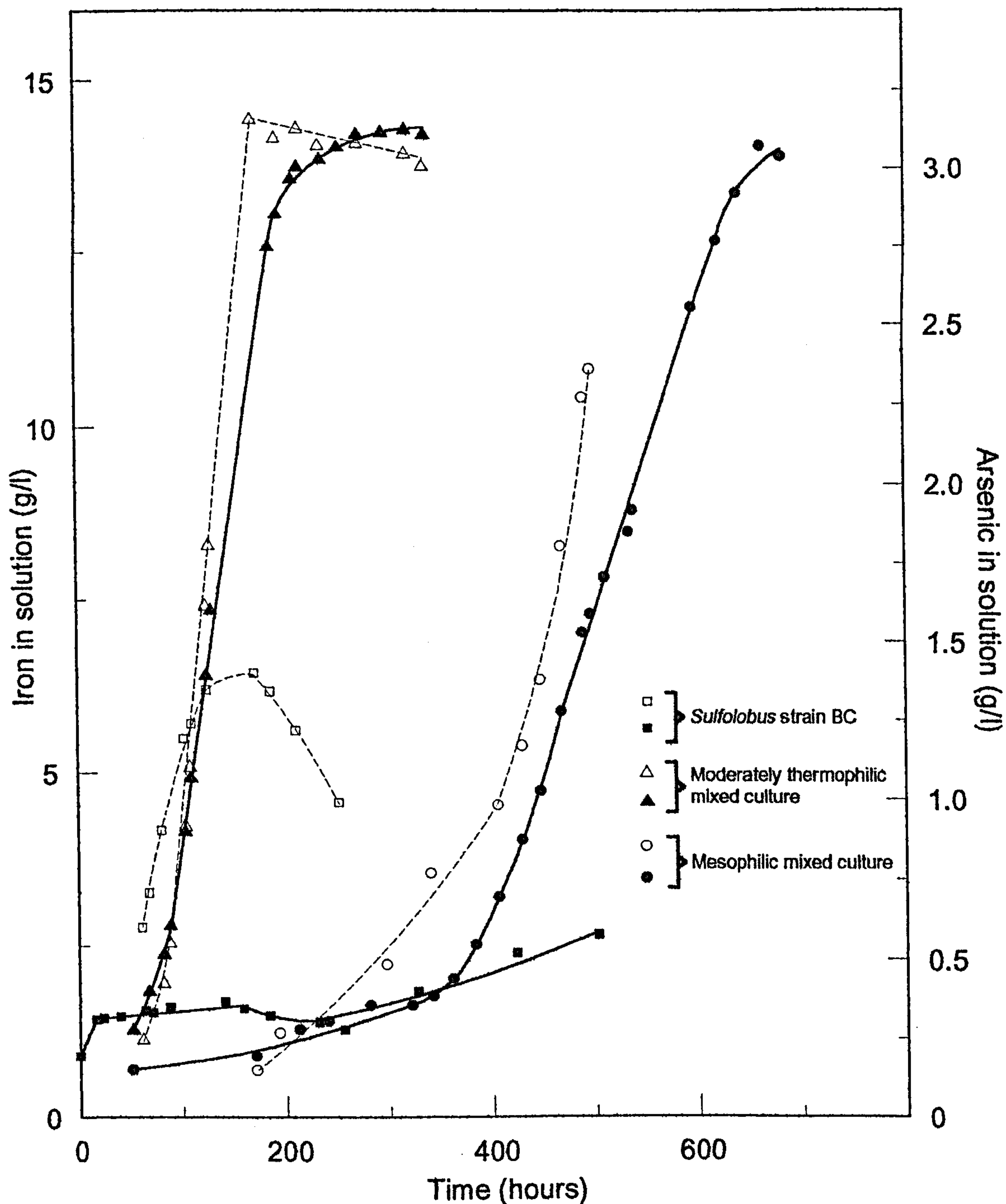


Figure 6.3: The solubilisation of 10% (w/v) Bogosu arsenopyrite concentrate during growth of three mineral-oxidizing cultures; a mesophilic mixed culture grown at 30 °C, a moderately thermophilic mixed culture grown at 48°C, and the extreme thermophile *Sulfolobus* strain BC grown at 68°C. The mesophilic culture was gassed with air, the moderately thermophilic culture and *Sulfolobus* strain BC were gassed with 1% (v/v) CO₂ in air. Open symbols and dashed lines represent arsenic concentrations; closed symbols and solid lines represent iron concentrations.

concentrate may be caused by the sensitivity of *Sulfolobus* species to agitation at high mineral pulp densities (Lindström *et al.*, 1992; Norris and Owen, 1993). Alternatively the level of arsenic in solution, 1.5 g/l after 170 hours, may have been inhibitory.

The mesophilic culture had a much increased time period prior to maximal solubilisation rates than on 2% (w/v) solids, from 40 hours to 300 hours. The maximal rate of solubilisation was greater at 44 mg/l/h iron release and 36 mg/l/h sulphate production, with a total mineral dissolution of 60-70%; again with a discrepancy between the values obtained from sulphate and iron solubilisation. Towards the end of the experiment, the solubilisation rate was still tailing off, suggesting that some more mineral dissolution would have taken place. The pH dropped to a final value of 1.43, and the ferrous iron remained between 100-140 mg/l after an initial increase. The last arsenic measurement was 2.4 g/l, but the final value, if solubilisation had continued in proportion to iron solubilisation, would have been approximately 2.75 g/l. Although the culture did not appear to be inhibited by this arsenic level, this was not altogether unexpected, as the levels obtained were much lower than reported inhibitory levels for mesophilic cultures of between 10-26 g/l (Morin and Ollivier, 1989; Bruynesteyn, 1993).

No great increase in the time period prior to maximal solubilisation was seen with the moderately thermophilic culture at the increased solid concentration. The maximal solubilisation rates were higher than on 2% (w/v) mineral concentrate, just as with the mesophilic culture. The rates were approximately twice those seen with the mesophilic culture at 89 and 288 mg/l/h for iron release and sulphate production respectively. The total iron and sulphate solubilisation indicated 71-75% mineral dissolution, at which point the culture had clearly become inhibited. This inhibition is likely to be caused by the low final pH of 1.02. The arsenic concentration may have become inhibitory, but this seems unlikely as moderately thermophilic cultures are known to remain active up to levels of 25 g/l arsenic (Spencer *et al.*, 1989; Lindström *et al.*, 1992).

To establish whether the extreme thermophile *Sulfolobus* strain BC was inhibited by high pulp density, or arsenic levels, fed batch experiments were carried out with this mineral concentrate. The culture was grown initially on 2% (w/v) Bogosu concentrate. Once leaching of this mineral had taken place, a further 2% (w/v) concentrate was added. Small increments in mineral were continued up to a total addition of 12% (w/v) concentrate (Figure 6.4).

In this fed batch system the extreme thermophile *Sulfolobus* strain BC leached a total of 12% (w/v) Bogosu concentrate. It seems reasonable to conclude from this that an initial pulp density of 10% (w/v) was the only reason *Sulfolobus* strain BC failed to grow in the previous experiment, and not because of the levels of arsenic in solution. The last arsenic measurement was 2.6 g/l, but the total arsenic in solution could have been approaching 4.5 g/l (if proportional to iron solubilisation values). This arsenic level is greater than that reported as being inhibitory to some extremely thermophilic mineral oxidizing cultures (Duarte *et al.*, 1993). The large ferrous iron peak of over 3 g/l seen upon addition of 2% (w/v) concentrate at approximately 200 hours, suggests that the initial small amount of mineral dissolution after this point was a result of indirect ferric iron leaching (Lundgren and Silver, 1980). However, this ferrous iron was rapidly oxidized, whilst total iron solubilisation increased to the maximal rate, indicating that bacterial activity was important for high rates of mineral dissolution. Upon all subsequent additions of mineral an initial increase in ferrous iron was followed by a drop back to a base level, indicating bacterial oxidation. The result of mineral additions at 350 and 400 hours suggests that maximal iron solubilisation rates could be maintained in this fashion and that a continuous leaching system would appear to be worthwhile.

The maximal iron solubilisation rates and total mineral dissolution in this fed batch system was close to those during growth on 2% (w/v) Bogosu concentrate, between 70-80 mg/l/h, and 70-76% respectively. The overall result being similar to that obtained with the moderately thermophilic culture on 10% (w/v) concentrate, producing almost identical rates and total mineral dissolution.

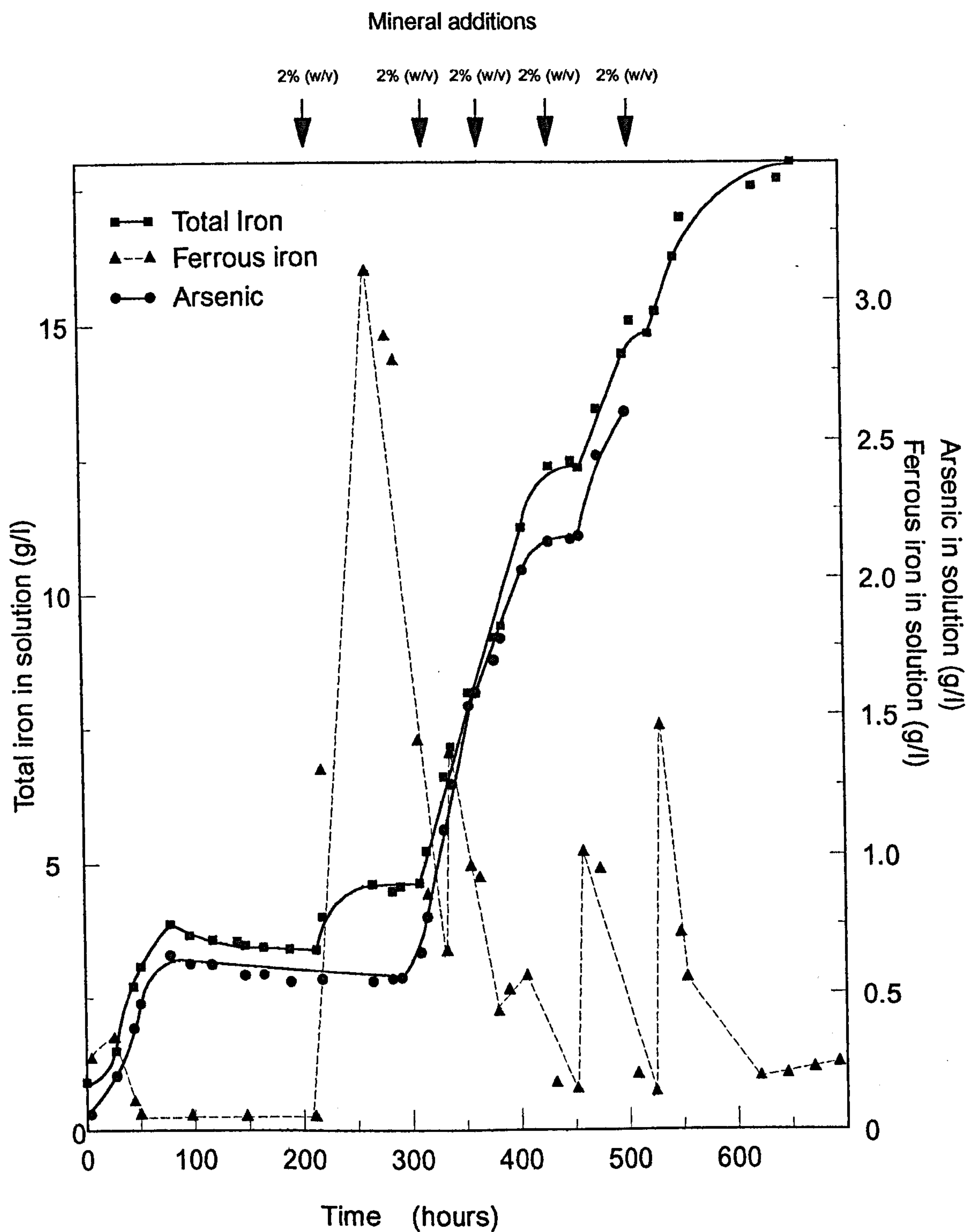


Figure 6.4: The solubilisation of 12% (w/v) Bogosu arsenopyrite concentrate during growth of *Sulfolobus* strain BC at 68°C. Mineral was added in 2% (w/v) increments at the points indicated by the arrows. The culture was gassed with 1% (v/v) CO₂ in air.

Fed batch experiments were carried out with the moderately thermophilic culture. The results were similar to those with *Sulfolobus* strain BC, with the moderately thermophilic culture still active at 12% (w/v) concentrate, suggesting that an arsenic level of 4.5 g/l was not inhibitory to this culture either.

6.4: Leaching of the Olympia arsenopyrite Concentrate.

There have been several laboratory evaluations using the Olympia concentrate from Greece (Lindström *et al.*, 1992). Mesophilic, moderately thermophilic and extremely thermophilic cultures have all been tested, but not in direct comparison. 2% (w/v) concentrate was used initially in a batch experiment. Once again the moderately thermophilic culture performed better than the other two cultures on this mineral, with the extreme thermophile *Sulfolobus* strain BC appearing unable to grow, and the mesophilic culture having a very long time period before maximal solubilisation occurred (Figure 6.5). One particular problem that arose with this concentrate that did not occur with the other two, was the difficulty in maintaining the mineral in suspension. Only by doubling the gas flow rate to 800 ml/min could the mineral be suspended. This increased gas flow may have resulted in more severe conditions (i.e. shear stresses) that could have prevented *Sulfolobus* strain BC becoming established.

The mesophilic culture took between 200-400 hours to reach maximal solubilisation rates, after which time the maximum iron release and sulphate production rates were 11.25 and 25.5 mg/l/h respectively. The difference between iron and sulphate derived total mineral dissolution values, seen with the Bogosu arsenopyrite, also occurred with this concentrate, with values of 92% total mineral dissolution by iron solubilisation and 63% by sulphate production. The final pH reached 1.30. A total of 1.7 g/l arsenic was released into solution, 85% of the total arsenic.

The moderately thermophilic culture again showed a maximal solubilisation rate much greater than the mesophilic culture, approximately three times the rate, 33.9 mg/l/h for iron release, and 91.5 mg/l/h for sulphate production. Mineral dissolution was complete, with a final pH of 1.22, and a final arsenic level of 2 g/l in

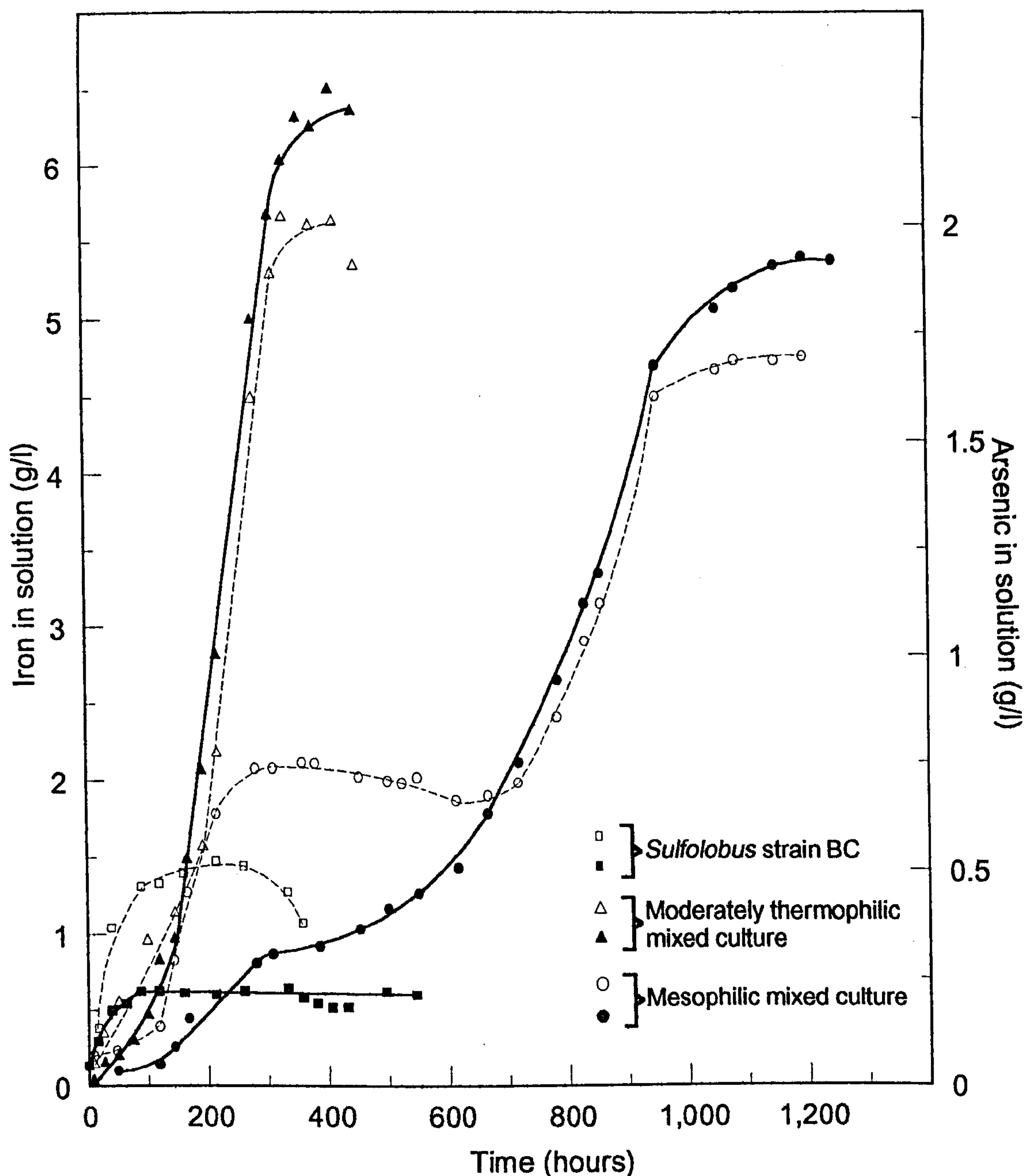


Figure 6.5: The solubilisation of 2% (w/v) Olympia arsenopyrite concentrate during growth of three mineral-oxidizing cultures; a mesophilic mixed culture grown at 30°C, a moderately thermophilic mixed culture grown at 48°C, and the extreme thermophile *Sulfolobus* strain BC grown at 68°C. The mesophilic culture was gassed with air at a rate of 800 ml/min, the moderately thermophilic culture and *Sulfolobus* strain BC were gassed with 1% (v/v) CO₂ in air at 800 ml/min. Open symbols and dashed lines represent arsenic concentrations; closed symbols and solid lines represent iron concentrations.

solution. The rate of iron solubilisation obtained here was slightly better than that obtained by Carlson *et al* (1992), who used a moderately thermophilic mixed culture obtained originally from this laboratory to leach 1.5% (w/v) Olympia mineral in stirred tank reactors. The arsenic levels reached here were still much lower than reported inhibitory levels.

As stated earlier, the inability of *Sulfolobus* strain BC to grow on 2% (w/v) Olympia mineral may have been a result of agitation at this solid concentration. It had been previously reported that growth of *Sulfolobus* strain BC did not occur on this mineral above 1.5% (w/v) concentrate in stirred tank reactors (Lindström *et al.*, 1992). In an attempt to obtain growth on Olympia concentrate above this value, cultures were grown initially on 1% (w/v) Olympia mineral concentrate mixed with 1% (w/v) Mexican pyrite concentrate (Figure 6.6). This mixture of the two minerals did result in leaching by *Sulfolobus* strain BC. Iron and arsenic were released into solution proportionally from the beginning of the experiment, and at such a level that both minerals must have been oxidized simultaneously. Between 86-92% total mineral dissolution occurred, with maximum dissolution rates of 93 mg/l/h for iron release and 330 mg/l/h for sulphur production.

The mesophilic and moderately thermophilic cultures produced almost identical leaching curves on this mixture of concentrates. Both gave 80-85% mineral dissolution, and had maximal solubilisation rates of 30-36 mg/l/h for iron release, and 99-117 mg/l/h for sulphate production, approximately one third of the rates seen with *Sulfolobus* strain BC. Again iron and arsenic were released into solution proportionally in amounts indicating simultaneous oxidation of the two minerals.

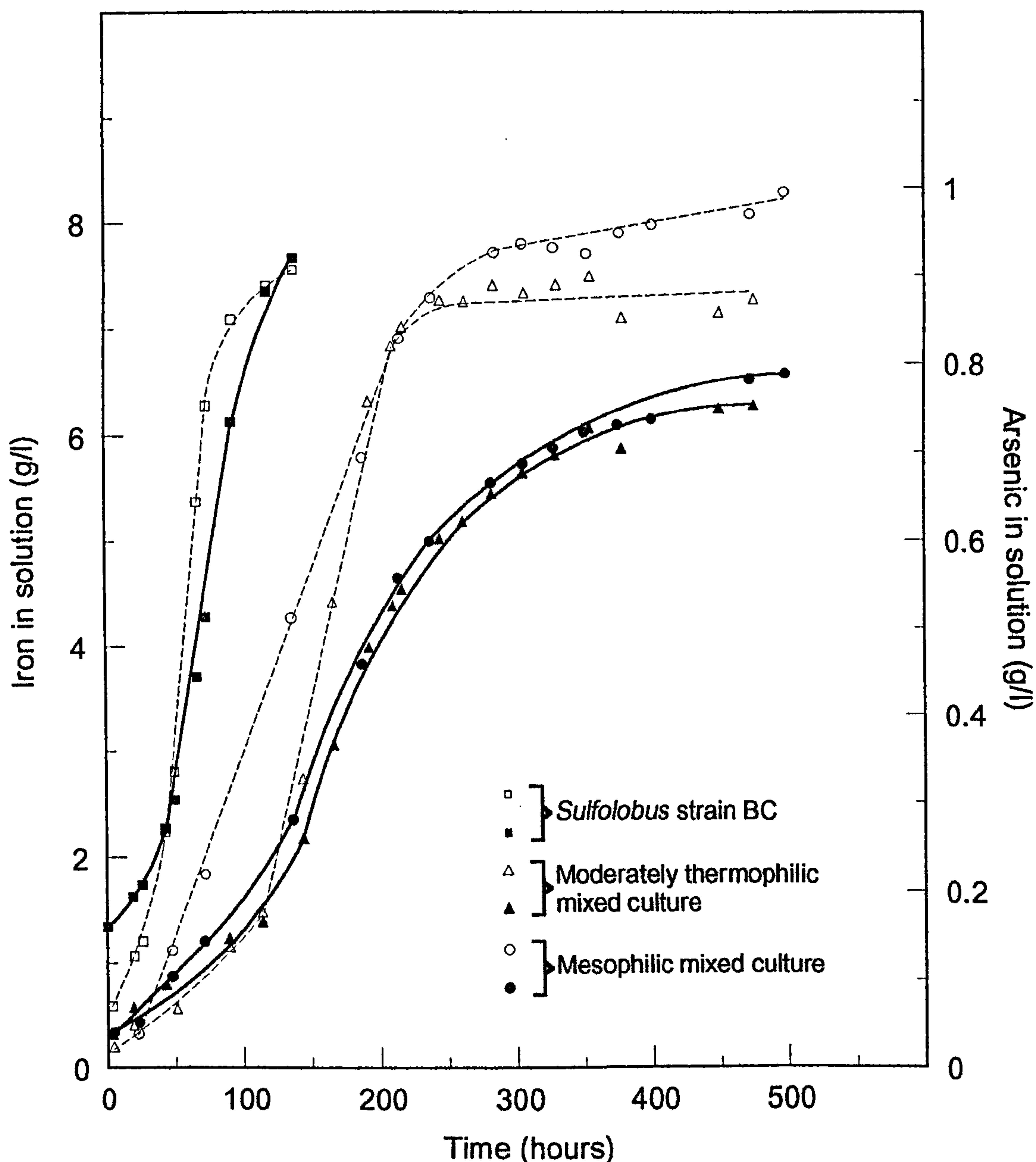


Figure 6.6: The solubilisation of a mixture of 1% (w/v) Olympia arsenopyrite concentrate and 1% (w/v) Mexican pyrite concentrate during growth of three mineral-oxidizing cultures; a mesophilic mixed culture grown at 30°C, a moderately thermophilic mixed culture grown at 48°C, and the extreme thermophile *Sulfolobus* strain BC grown at 68°C. The mesophilic culture was gassed with air at a rate of 800 ml/min, the moderately thermophilic culture and *Sulfolobus* strain BC were gassed with 1% (v/v) CO₂ in air, at 800 ml/min. Open symbols and dashed lines represent arsenic concentrations; closed symbols and solid lines represent iron concentrations.

Once *Sulfolobus* strain BC had become established on this mixture of minerals, leaching of further additions of 2% (w/v) Olympia mineral were possible (Figure 6.7). Each addition gave a characteristic initial peak in ferrous iron, and then a subsequent fall to a base level, indicating bacterial activity. A total of 5% (w/v) Olympia concentrate was added, with 90-100% mineral dissolution. Although the last arsenic measurement made was only 2.2 g/l, the estimated arsenic level for this amount of mineral dissolution would be 4.5-5 g/l (providing arsenic was released in proportion to iron), at which level the bacteria were still active.

Although arsenic concentrations reached levels of up to 5 g/l, they never approached levels likely to be inhibitory in any of these experiments. The data therefore, provides some useful rate comparisons between the cultures on different minerals, but does not give any real indications of differences in arsenic resistances.

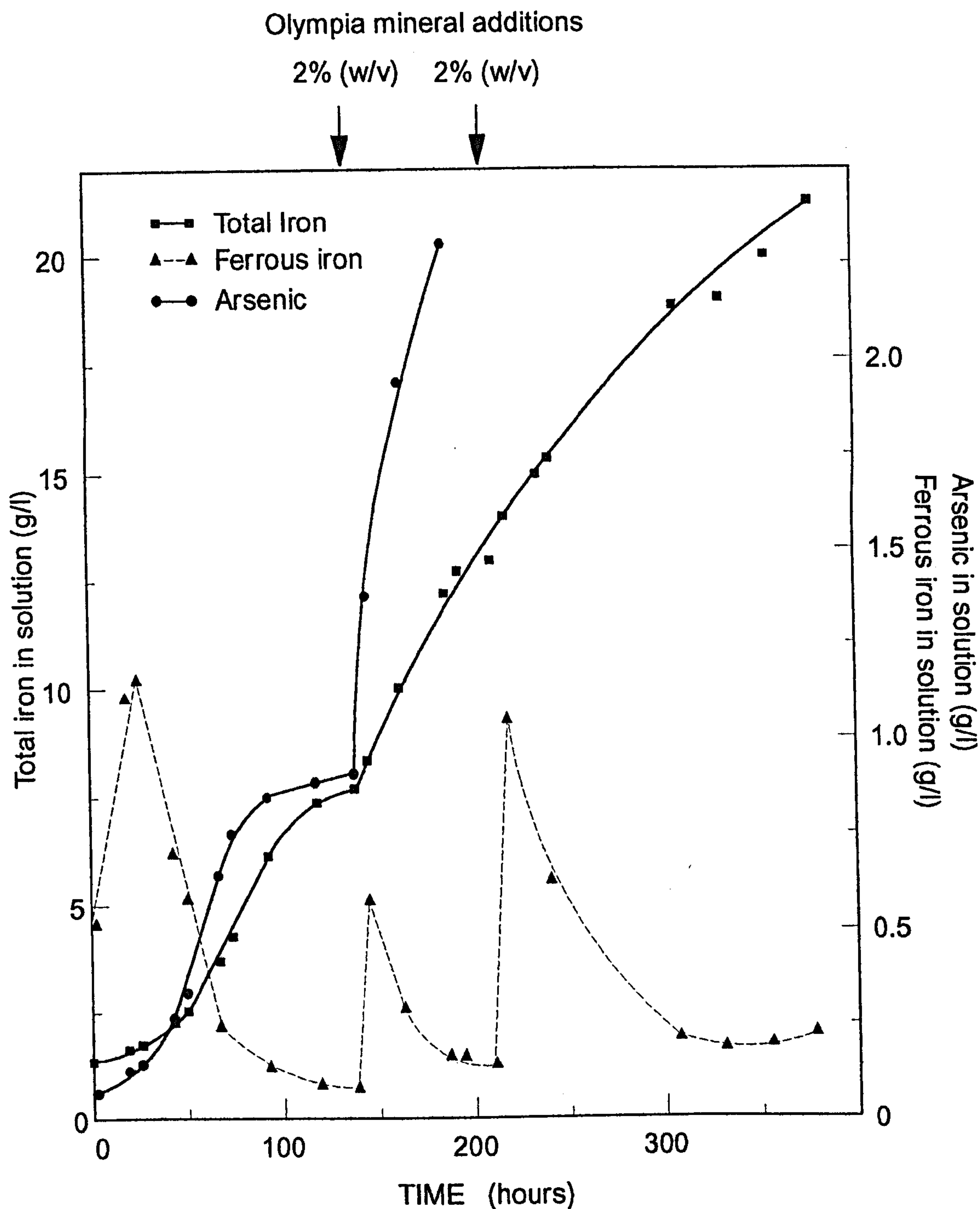


Figure 6.7: The solubilisation of 4% (w/v) Olympia arsenopyrite concentrate during growth of *Sulfolobus* strain BC at 68 °C, after initial growth on a mixture of 1% (w/v) Mexican pyrite and 1% (w/v) Olympia arsenopyrite. Olympia mineral was added in 2% (w/v) increments at the points indicated by the arrows. The culture was gassed with 1% CO₂ (v/v) in air, at a rate of 800 ml/min.

6.5: Mineral leaching by an Icelandic moderately thermophilic mixed culture.

Despite the promising leaching results obtained here with the moderately thermophilic mixed culture, one of the main disadvantages of commercial application of these organisms over the mesophilic cultures currently used, is their requirement for enhanced CO₂ levels, a potential increase in process cost and complexity. A novel mixed culture was isolated from an Icelandic thermal spring, cultured and partially characterised within this laboratory (see chapter 4). It was subsequently found to oxidize mineral sulphides at temperatures between 45-50 °C without the requirement for enhanced CO₂ levels (Norris and Owen, 1993). In order to establish the potential of this novel culture in mineral-oxidation, comparisons were made with the previously used moderately thermophilic culture on the three test minerals.

During the leaching of 2% (w/v) Mexican pyrite concentrate the performance of the novel mixed culture grown in air, was found to be slightly better than the previous moderately thermophilic mixed culture grown at enhanced CO₂ levels, and vastly superior to it when it was grown in air (Figure 6.8). Fig. 6.8 also shows the growth of moderately thermophilic strain BC1 at enhanced CO₂ levels. This pure culture gave very similar results to the mixed culture used previously at enhanced CO₂ levels. The novel Icelandic mixed culture gave a maximum iron solubilisation rate of 61 mg/l/h compared to 44 mg/l/h for both the original mixed culture and strain BC1 (grown at enhanced CO₂ levels). Total mineral dissolution by the novel Icelandic culture was also greater at 87%, and the final pH lower at 1.04. This may be an indication that this culture has a higher acid tolerance than previously studied moderately thermophilic bacteria.

Very similar results were obtained with the novel Icelandic mixed culture during the leaching of 2% (w/v) Bogosu arsenopyrite concentrate in air as were obtained previously at enhanced CO₂ levels by the original moderately thermophilic mixed culture (Figure 6.9). Both cultures giving maximal iron solubilisation rates of 27-30 mg/l/h, but the Icelandic mixed culture resulted in about 5% greater total mineral dissolution at 90%, compared to 85% with the previous culture.

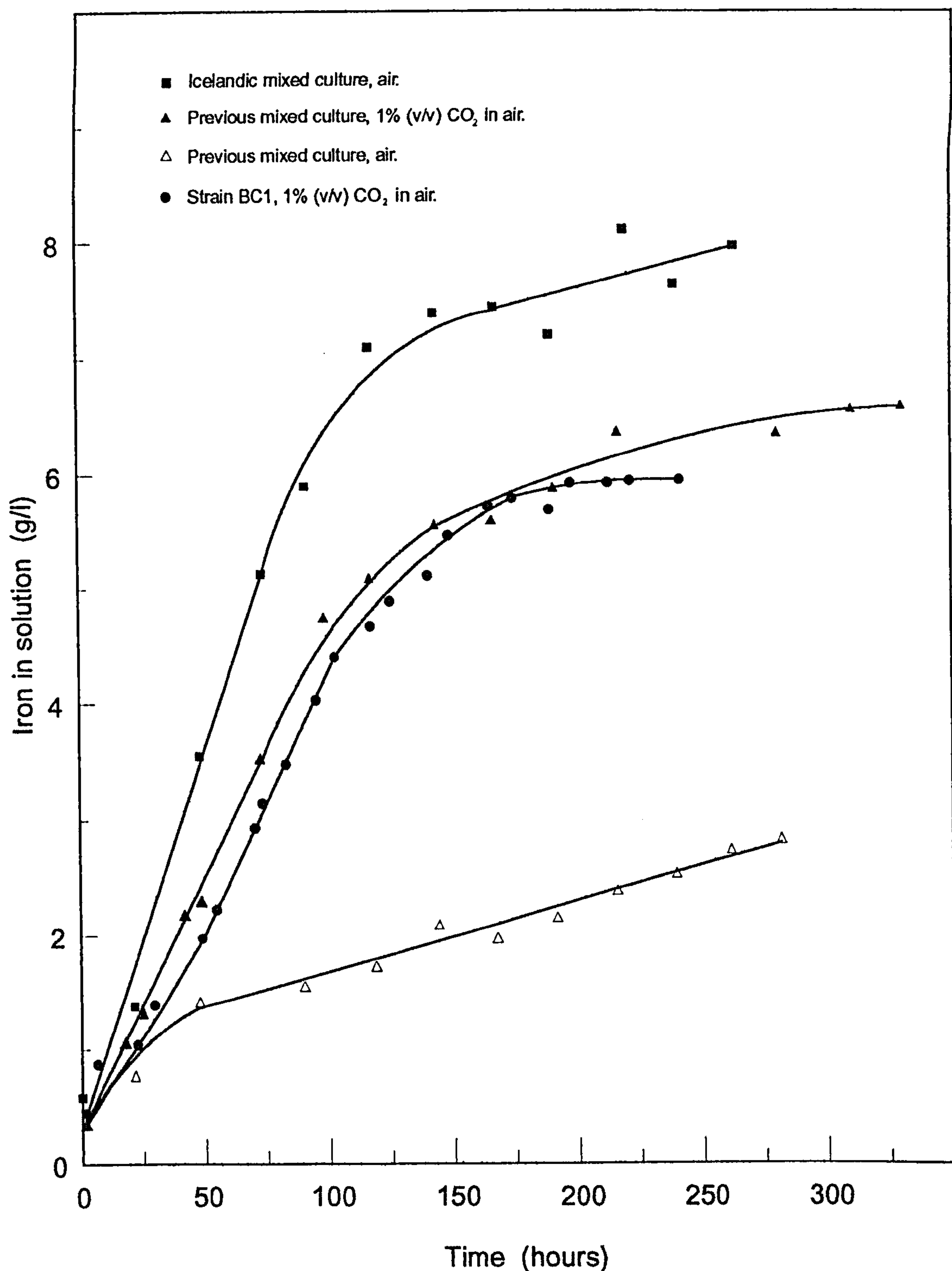


Figure 6.8: The solubilisation of 2% (w/v) Mexican pyrite concentrate during growth of three moderately thermophilic cultures at 48°C: a mixed culture obtained from Iceland, gassed with air, the mixed culture used in the previous leaching studies, gassed with air or 1% (v/v) CO₂ in air, and strain BC1, gassed with 1% (v/v) CO₂ in air.

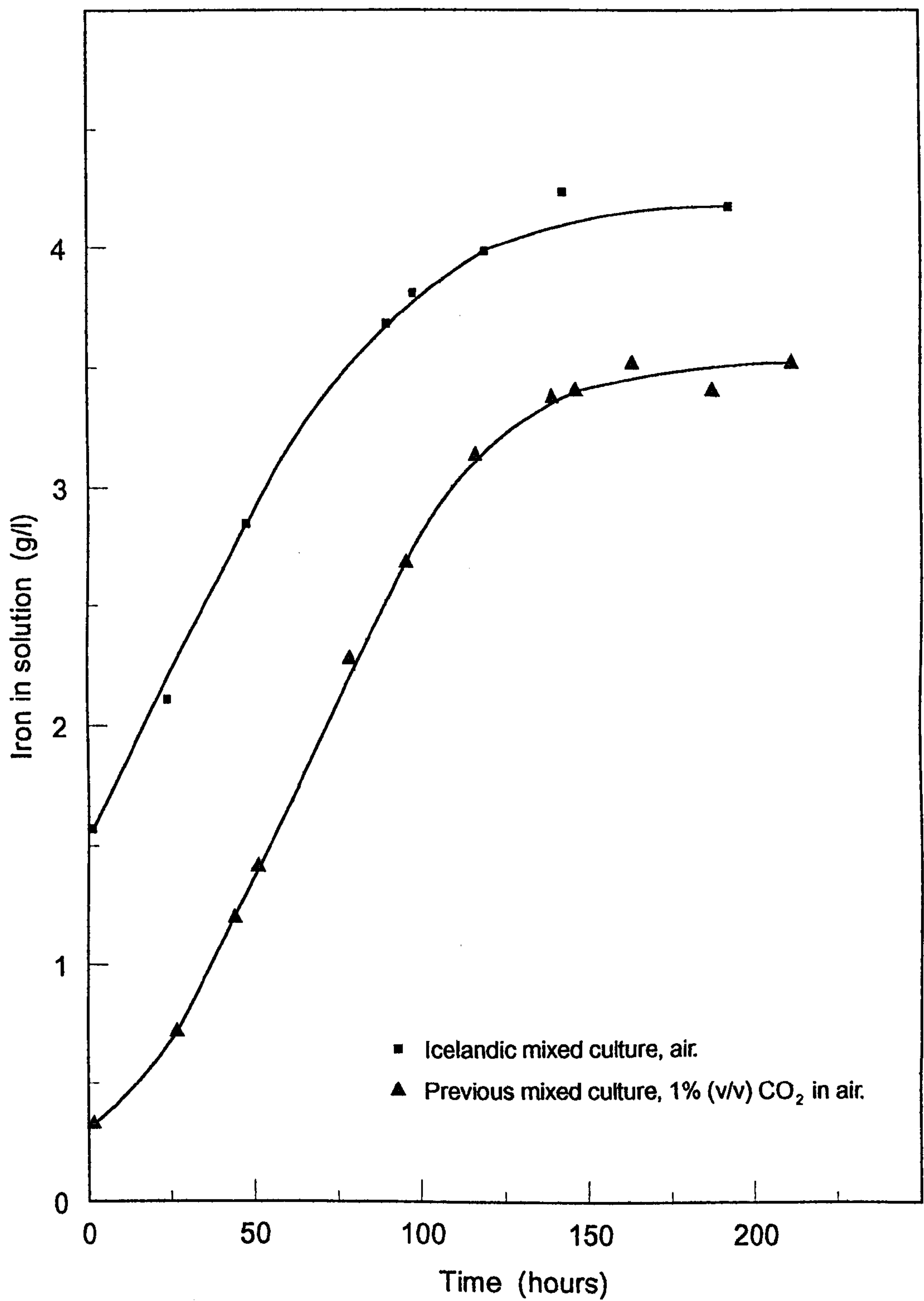


Figure 6.9: The solubilization of 2% (w/v) Bogosu arsenopyrite concentrate during growth of two moderately thermophilic mixed cultures at 48°C: a mixed culture obtained from Iceland, gassed with air, and the mixed culture used in the previous leaching studies, gassed with 1% (v/v) CO₂ in air.

A similar trend was seen during growth on 2% (w/v) Olympia concentrate (Figure 6.10). The novel Icelandic culture gave a maximal iron solubilisation rate of 40 mg/l/h, and a total mineral dissolution of 96%. This compares favourably with the results obtained with the original mixed culture, 30 mg/l/h and 80-85% mineral dissolution. The novel Icelandic culture also gave a lower final pH of 1.08.

The results obtained with this novel mixed culture suggest that it has great potential in mineral leaching. It was capable of growth on all three minerals with higher or equal solubilisation rates to the previously studied moderately thermophilic culture, and consistently gave higher total mineral dissolution levels. This culture, therefore, appears not only to give better mineral dissolution results, but also appears to have a greater acid tolerance and grows without an enhanced CO₂ concentration.

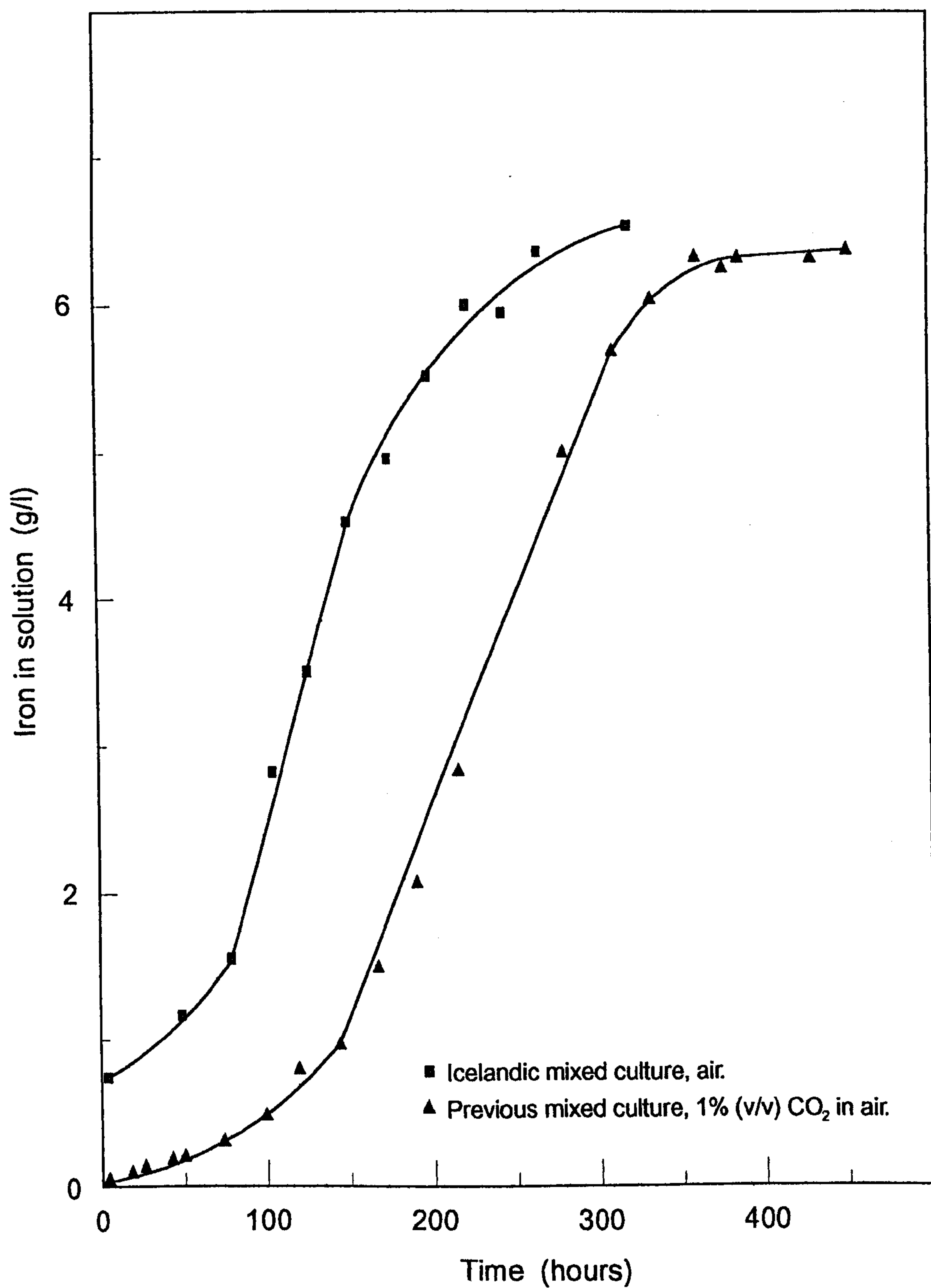


Figure 6.10: The solubilisation of 2% (w/v) Olympia arsenopyrite concentrate during growth of two moderately thermophilic cultures at 48°C: a mixed culture obtained from Iceland, gassed with air, and the mixed culture used in the previous leaching studies, gassed with 1% (v/v) CO₂ in air.

6.6: Discussion.

The results obtained here clearly indicate that, in general increasing the temperature during leaching of a mineral will produce higher rates of mineral dissolution. However, each situation must be evaluated carefully, as results vary with different minerals. For example, the initial results obtained with *Sulfolobus* strain BC suggested that it would be of limited use in the oxidation of Olympia concentrate, but the results with the mixture of the Mexican pyrite and Olympia arsenopyrite clearly showed the potential of this organism.

The data obtained here give no indication of the precious metal recoveries after bacterial oxidation, an essential piece of information before any culture or process could be selected. However, gold liberation from refractory ores is generally proportional to the amount of mineral dissolution (Lindström *et al.*, 1992). Mineral dissolution comparisons can therefore give some indication of the likely metal recoveries and thus the potential of each culture.

The benefits of the extreme thermophile were most clearly seen during growth on 2% (w/v) Bogosu arsenopyrite, and on the mixture of Mexican pyrite with Olympia arsenopyrite. In both cases the maximal rates of iron solubilisation were 2-3 fold greater than with the other cultures. The main problem encountered with the extreme thermophile *Sulfolobus* strain BC was its apparent sensitivity to agitation at high pulp densities, as has been suggested by previous work (Lindström *et al.*, 1992). However enrichment cultures may provide a means by which this problem could be avoided, as was the case with the moderately thermophilic organisms and their requirement for enhanced CO₂ levels. Indeed an extremely thermophilic culture may have already been found that is capable of withstanding agitation at higher pulp densities (Norris and Owen, 1993). It should also be noted that although enhanced CO₂ levels were supplied to the extreme thermophile during these experiments, it is not a necessary requirement for growth (Norris, 1989), rather it simply enhances the rates of dissolution.

The potential offered by the extreme thermophiles to mineral leaching is probably the greatest of the three cultures, but their full scale application is some time

away. Various factors limit their commercial application including: inactivation below 55 °C, sensitivity to agitation at high pulp density, and the adequate supply of CO₂ and oxygen at elevated temperatures (Brierley, 1993).

Overall the moderately thermophilic culture produced the best all round performance on the minerals tested. Rates of mineral dissolution were consistently greater than with the mesophilic culture even at the higher concentration of solids (10% (w/v)), and no growth problems occurred in comparison to those with *Sulfolobus* strain BC.

The application of moderately thermophilic organisms is at a much closer stage to commercialisation than the extreme thermophiles. Several of the commercial reactors for bio-oxidation of refractory gold ores now operate at temperatures between 40-45 °C (van Aswegen, 1993), a range where moderately thermophilic bacteria are active. The non-sterile nature of these processes does not preclude the presence of moderately thermophilic organisms (Brierley, 1993). An increase in operating temperature to 45-50 °C is not inconceivable. The exothermic nature of mineral dissolution would mean that little or no further energy input would be required. Indeed the Fairview bio-oxidation process is known to peak at a maximum temperature of 53 °C for brief periods, and an increase in running temperature from 40 to 45 °C has been estimated to reduce cooling costs by 20-30% (van Aswegen *et al.*, 1991). Bactech (Australia) Ltd. have tested a moderately thermophilic culture at a number of sites in Australia with encouraging results (Budden and Spencer, 1993).

One of the main inadequacies of the moderate thermophiles, their CO₂ requirement, appears to have been overcome by the use of the novel Icelandic mixed culture. This culture produced mineral dissolution rates, when grown in air, equal to or better than those obtained with the previously studied moderately thermophilic mixed culture grown at 1% (v/v) CO₂ in air. Indications are that this culture also has an increased acid tolerance further strengthening its commercial potential.

In conclusion, the results suggest that thermophilic organisms offer an attractive alternative to present bio-oxidation pre-treatment of refractory precious metal ores.

They have the potential for enhanced oxidation rates, and can reduce process cooling costs.

Chapter 7:
Conclusions.

Data from three main areas of work has been presented in this thesis, all relating to the potential commercial application of moderately thermophilic cultures:

1. The characterisation and classification of moderately thermophilic isolates.
2. The characterisation of the constituent organisms and interactions within a novel mixed culture.
3. The comparison of thermophilic and mesophilic leaching of mineral concentrates in bioreactors.

A number of the moderately thermophilic strains have been characterised. DNA:DNA hybridisation studies and protein profile analysis both indicated three distinct groups of organisms (see Fig. 3.2 and 3.4). The first group includes the only isolate so far named, *Sulfobacillus thermosulfidooxidans*. This group also contains several other well studied isolates from around the world (including strains BC1 and TH1), suggesting that this type of organism is widespread within mineral leaching environments. The second group is typified by strain NAL, but isolates from this group come from a variety of sites around the world, suggesting that these organisms are also widespread in nature. At the beginning of this study, the third group consisted of a single isolate, strain TH3. Strain TH3 had only ever been isolated from a single site in New Mexico, and so was not believed to be widespread in nature. However, the isolation of a novel strain (ICP) that was closely related to strain TH3 (see section 4.3) suggests that this type of moderately thermophilic iron-oxidizer is more widespread than previously believed. On the basis of the DNA:DNA hybridisation values these three groups should receive separate genus status.

Various morphological and physiological data support the separation of these organisms into three groups. The three groups differ in their heterotrophic, and autotrophic growth characteristics, their comparative resistances to heavy metals and antibiotics, and their cell morphologies during growth on different substrates (see sections 3.5 and 3.6).

These results should provide a firm base for examination of novel strains, and should eliminate any confusion over which organisms are being used by different laboratories. The importance of this work is clearly indicated by the apparently wrong classification of *S. thermosulfidooxidans* as a close relative of *Alicyclobacillus* in the literature (Tourova *et al.*, 1994).

Characterisation of a novel mixed culture from Iceland indicated that two organisms were necessary for good autotrophic growth under air (see chapter 4). The key organism within this mixed culture (strain ICP) possessed a high affinity CO₂ uptake system, and sufficient regulation of RuBisCO to support autotrophic growth under air (see chapter 5), but a higher affinity for ferric iron than ferrous iron during autotrophic growth in pure culture, limited this organism's iron oxidation capabilities (see section 4.4.3). The presence of a *Sulfobacillus*-type organism within the mixed culture enabled high rates of iron oxidation to continue. This second organism probably utilises fixed carbon from strain ICP. The continued high rates of iron oxidation seen with the mixed culture may be a result of oxidation by the second organism alone, or alternatively the presence of a *Sulfobacillus*-type organism may improve the health of strain ICP, and iron oxidation by both organisms would continue.

The possession of a high affinity CO₂ uptake system had not been previously demonstrated in moderately thermophilic iron-oxidizing bacteria (Norris, 1989b), but the results obtained here, and previously (MacLean, 1993; Holthuijzen *et al.*, 1987; Karagouni and Kelly, 1989), indicate that such a system is essential for autotrophic growth of both moderate thermophiles and thiobacilli under air.

Further molecular characterisation of CO₂ fixation by strain ICP, indicated that in common with strain NAL its RuBisCO was the typical hexadecameric L₈S₈ form I enzyme (see chapter 5). Comparison of the nucleotide and deduced amino acid sequences of a fragment of the RuBisCO LSU gene (amino acid residues 70-200 according to spinach numbering) indicated that strain ICP had greater homology to RuBisCO LSU genes from photosynthetic origin than to those of chemolithotrophic origin (see Fig. 5.14). In contrast the same gene fragment from strain NAL showed

greater homology to the RuBisCO LSU of the chemolithotroph *Alcaligenes eutrophus* (see Fig. 5.14). This suggests a difference in the evolution of the RuBisCO molecule amongst these moderate thermophiles. Hypothetical schemes for the evolution of RuBisCO have been proposed (McFadden *et al.*, 1986), in which photosynthetic bacteria are believed to be the common ancient ancestor. If such a scheme is correct then the greater homology of the RuBisCO of strain ICP to RuBisCO of photosynthetic origin suggests that strain ICP is a more ancient type of moderately thermophilic iron-oxidizer than the *Sulfobacillus*-type organisms.

The potential of this novel mixed culture in mineral leaching was clearly demonstrated (see section 6.5). This mixed culture produced oxidation rates on all three test minerals equal to those obtained previously with moderately thermophilic cultures at enhanced CO₂ levels (Fig. 6.8, 6.9, and 6.10).

Precious metal recovery values were not measured during the mineral leaching studies, and these will ultimately be the values that commercial processes will depend upon. However, it is generally accepted that precious metal recovery is usually proportional to mineral dissolution (Lindström *et al.*, 1992), and so the mineral dissolution rates obtained should have relevance to commercial application.

The mineral leaching studies indicated that mineral dissolution rate increased in proportion to the temperature. The extreme thermophile *Sulfolobus* BC produced the best maximal rates of mineral dissolution at low solid concentration (see chapter 6), but produced poor rates of dissolution at higher solid concentrations, as had been found previously (Lawrence and Marchant, 1988; Lindström and Gunneriusson, 1990). For industrial application of the extreme thermophiles, mineral leaching at higher solid concentrations is needed. Enrichment cultures may in the future produce extremely thermophilic organisms with improved resistance to agitation at high solid concentrations (Norris and Owen, 1993), and thus leaching at increased pulp densities would be possible.

A second reported limitation of the extreme thermophiles is their low resistance to toxic metals, such as arsenic. However, in a fed batch leaching system *Sulfolobus* BC

remained active at estimated arsenic levels of between 4-5 g/l (Fig. 6.4). This is 2-3 times higher than toxic values that have been reported in other studies (Duarte *et al.*, 1993).

The moderately thermophilic mixed culture produced the best overall performance on the range of minerals tested (Fig. 6.1, 6.2, and 6.5). This takes account of the fact that this culture was capable of mineral dissolution rates as good as or better than the mesophilic culture on all the minerals, even at the higher solid concentration of 10% (w/v) (Fig 6.3), and particularly appeared more tolerant of high solids than the potentially faster extreme thermophile. This may have important commercial consequences. Indications from industry are that bio-oxidation plants already operate at temperatures between 40-45 °C, and can peak as high as 53 °C (van Aswegen *et al.*, 1991). This temperature is at the limit (or above that) of the growth range for mesophilic organisms, but is a temperature at which moderate thermophiles are most active. The leaching studies carried out here indicate that the use of moderate thermophiles would seem to be a logical step forward from the use of mesophiles. Indeed the isolation of moderately thermophilic organisms from laboratory scale continuous processes (Goebel and Stackebrandt, 1994) suggests that these organisms may already be an important part of the mixed cultures used in bioleaching.

These data clearly indicate the potential of moderately thermophilic cultures in commercial bioleaching. If these organisms are not already involved in commercial operations, then their introduction would be relatively simple to achieve. They offer improved dissolution rates over those achieved with mesophiles on a range of minerals. Even the requirement for enhanced CO₂ levels appears to have been overcome with the discovery of a novel mixed culture. Furthermore, the characterisation of the major groups of moderately thermophilic iron-oxidizing bacteria that has been carried out, in conjunction with 16S rRNA data should enable the rapid identification of these organisms, with an expected increase in the recognition of the role they play in mineral leaching.

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